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IDENTIFICATION AND CHARACTERIZATION OF STEM LEYDIG CELLS IN THE TESTIS

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Identification and Characterization of Stem Leydig Cells in the Testis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

In the testis, several cell types are needed to ensure male fertility, among them the testosterone-producing Leydig cells. Testosterone supports the maturation of sperm and maintains the male phenotype. Functional Leydig cells are therefore required to prevent testicular malfunction. Yet in contrast to their well-established role in the testis, the origin of the adult Leydig cell lineage has largely remained unclear. A potential reservoir for putative stem Leydig cells are the peritubular myoid cells, which show characteristics of pluripotency as well as steroidogenic capacity and can be isolated to study the differentiation mechanisms of steroidogenic cells. The scope of this work is to elucidate cells and pathways involved in the underlying processes.

Common to all stages of Leydig cell development is the presence of the platelet-derived growth factor receptor alpha (PDGFRα). Stem Leydig cells have been described to be PDGFRα-positive and to not express the luteinizing hormone receptor (LHR), a classical marker for differentiated Leydig cells.
To identify the stem Leydig cell in the testis, we first analyzed the two steroidogenic cell types in the postnatal rodent testis. Both, the fetal Leydig cells and the peritubular cells were PDGFRα positive and showed a transient capacity to produce steroids. Stimulation of the cAMP-PKA pathway increased the steroidogenic potential in both cell types. However, while the FLCs were responsive to hCG stimulation, the PTCs expressed no LHR and their steroid production did not increase during hCG treatment. The isolated PDGFRα-positive, LHR-negative rat PTCs expressed markers for pluripotent, steroidogenic and myoid cells, indicating that they include putative stem Leydig cells.

Subsequently, we examined the transferability of the rodent data into humans. We found that the peritubular cells in the adult human testis (HTPC) also expressed the PDGFRα and showed characteristics of pluripotent as well as steroidogenic cells. Stimulation of the cAMP-PKA pathway in isolated HTPCs increased the steroid production, underscoring the similarities between rat and human Leydig cell development.

To be able to study early differentiation mechanisms in detail, the rapidly developing field of stem cell technology opens new possibilities. As the overexpression of the steroidogenic factor 1 (SF-1) has been described to induce the differentiation of steroidogenic cells from mesenchymal and embryonic stem cells, we characterized seven human embryonic stem cell lines with the aim of identifying similar factors. We found that all seven cell lines express different marker profiles for differentiating cells, with one cell line, *i.e.* HS361 showing characteristics of Sertoli-like cells and another cell line, *i.e.*HS420, expressing early differentiation marker for steroidogenic cells. Further characterization and differentiation of this cell line could increase our understanding of the development of steroidogenic cells.

In summary, this work shows that the peritubular cell population in the testis contains putative stem Leydig cells and that these cells can be stimulated to become steroidogenic. The cAMP-PKA pathway plays an important role in the maintenance of steroidogenesis and prevents dedifferentiation under *in vitro* conditions. We furthermore identified candidate human embryonic stem cell lines for the study of the underlying mechanisms of gonadal cell development.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals:

I. Judith Weisser *, **Luise Landreh** *, Olle Söder, Konstantin Svechnikov.
Steroidogenesis and steroidogenic gene expression in postnatal fetal rat Leydig cells.
Molecular and Cellular Endocrinology 341 (2011), p. 18-24

II. **Luise Landreh**, Jan-Bernd Stukenborg, Olle Söder, Konstantin Svechnikov.
Phenotype and steroidogenic potential of PDGFRα-positive rat neonatal peritubular cells.
Molecular and Cellular Endocrinology 372 (2013), p. 96-104

III. **Luise Landreh** *, Katrin Spinnler *, Kerstin Schubert, Merja Häkkinen, Seppo Auriola, Matti Poutanen, Olle Söder, Konstantin Svechnikov, Artur Mayerhofer.
Human testicular peritubular cells host putative stem Leydig cells with steroidogenic capacity.
The Journal of Clinical Endocrinology and Metabolism 99 (2014), p. 1227-35

IV. **Luise Landreh** *, Kristín Rós Kjartansdóttir *, Halima Albalushi, Olle Söder, Konstantin Svechnikov, Outi Hovatta, Jan-Bernd Stukenborg.
Characterization of seven human embryonic stem cell lines according to their differentiation potential towards somatic cells of the testis.
Manuscript

*: these authors contributed equally to the work

ADDITIONAL PUBLICATIONS (Not included in the thesis)

1. Konstantin Svechnikov, **Luise Landreh**, Judith Weisser, Gaia Izzo, Eugenia Colon, Irina Svechnikova, Olle Söder.
   Origin, Development and Regulation of Human Leydig Cells.
   Hormone Research in Paediatrics 73 (2010), p. 93-101

2. Konstantin Svechnikov, Gaia Izzo, **Luise Landreh**, Judith Weisser, Olle Söder.
   Endocrine Disruptors and Leydig Cell Function.
   Journal of Biomedicine and Biotechnology (2010)

3. Ahmed Reda, Mi Hou, **Luise Landreh**, Kristin Rós Kjartansdottir, Konstantin Svechnikov, Olle Söder, Jan-Bernd Stukenborg.
   In vitro spermatogenesis- optimal culture conditions for testicular cell survival, germ cell differentiation and steroidogenesis in rat.
   Frontiers in Experimental Endocrinology 26 (2014), p. 5-21

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3-βHSD, HSD3B, Hsd3b1	3-beta hydroxysteroid dehydrogenase, protein and gene names (human and rat)
17-βHSD, Hsd17b3	17-beta hydroxysteroid dehydrogenase, protein and gene names
Actb	Beta actin, gene name (rat)
ACTH	Adrenocorticotropic hormone
ALC	Adult Leydig cell
αSMA, Acta2	Smooth muscle actin, protein and gene names (rat)
AMH	Anti-mullerian Hormone
AR	Androgen receptor
BMP	Bone marrow protein
BSA	Bovine serum albumin
$(Bu)_2$cAMP	Di-Butyryl cyclic adenosine monophosphate
CD90/Thy-1	Cluster of Differentiation 90/ Thymus cell antigen 1
Cox 2	Cyclooxygenase 2
Ct	Cycle threshold
DAB	3,3'-Diaminobenzidine
DDX 4	DEAD box polypeptide 4 (Vasa), gen name (human)
Dhh	Desert hedgehog protein, protein name
DMEM	Dulbecco’s Modified Eagle’s Medium
DNMT3B	DNA (cytosine-5-) methyltransferase 3 beta, gene name (human)
E	Embryonic day
ECM	Extracellular matrix
EDS	Ethane dimethane sulphonate
ESC	Embryonic stem cell
FCS	Fetal calf serum
FGF2	Fibroblast growth factor 2
FLC	Fetal Leydig cell
FSH/ FSHR	Follicle stimulating hormone/ receptor
GABRB3	Gamma-aminobutyric acid receptor subunit beta-3, gene name (human)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, gene name (human)
Gata4/6, GATA4/6	GATA-binding protein 4/6, protein and gene names (human)
GDF 3	Growth differentiation factor 3
GFP	Green fluorescent protein
HBSS	Hank’s Balanced Salts Solution
hCG	Human chorionic gonadotropin, protein name
HTPC	Human testicular peritubular cell
HTPCF	Human testicular peritubular cell derived from patients with fibrotically remodeled testis

ICM	Inner cell mass
IHC	Immunohistochemistry
IL 1α/1β/2/6	Interleukin 1α/1β/2/6
ILC	Immature Leydig cell
Insl-3, INSL3, Insl3	Insulin-like factor 3, protein and gene names (human and rat)
IPSC	Induced pluripotent stem cell
LC	Leydig cell
LH	Luteinizing hormone, protein name
LHR, LHCGR, Lhcgr	Luteinizing hormone receptor, protein and gene names (human and rat)
LRH1, NR5A2	Liver receptor homologue 1, protein and gene names (human)
LIF	Leukaemia inhibitory factor, protein name
LIFR, Lifr	Leukaemia inhibitory factor receptor, protein and gene names (human and rat)
LN	laminin
LRH-1, NR5A2	Liver receptor homologue 1, protein and gene names (human)
MACS	Magnetic cell separation technique
MC2R	Melanocortin 2-receptor (ACTH receptor), gene name (human)
MEM	Modified Eagle's Medium
MS	Mass spectrometry
Myh11	Myosin heavy chain, gene name (rat)
Nes	Nestin, gene name (rat)
Oct3/4, Pou5f1, POU5F1, Pou5f1	Octamer-binding transcription factor ¾, protein and gene names (human and rat)
P450scc, CYP11A1, Cyp11a1	Cytochrome P450 with side chain cleavage activity, protein and gene names (human and rat)
P450c17, CYP17A1, Cyp17a1	Cytochrome 450 C17, protein and gene names (human and rat)
PAP 7	TSPO-associated protein 7
PBR, TSPO, Tspo1	Peripheral benzodiazepine receptor/ translocator protein, protein and gene names (human and rat)
PCR (RT-, q-)	Polymerase chain reaction (reverse transcriptase-, quantitative)
PDGF-A	Platelet derived growth factor A, protein name
PDGF-B	Platelet derived growth factor B, protein name
PDGFRα, PDGFRA, Pdgfra	Platelet derived growth factor receptor alpha, protein and gene names (human and rat)
PFA	Paraformaldehyde
PFLC	Postnatal fetal Leydig cell
PKA	Protein kinase A
PKAR1A	Protein kinase A regulatory subunit 1α

PLC	Progenitor Leydig cell
PTC	Peritubular cells
PSLC	Putative stem Leydig cells
RA	Retinoid acid
RIA	Radio immune assay
RPL19	Ribosomal protein L19, gene name (human)
SCF	Stem cell factor
SCID	Severe combined immune deficiency
SD rats	Sprague dawley rats
SER	Smooth endoplasmatic reticulum
SF-1, NR5A1, Nr5a1	Steroidogenic factor 1, protein and gene names (human and rat)
SLC	Stem Leydig cell
Sox 2/9/17	SRY-box 2/9/17
Sry	Sex-determining region Y chromosome
SSC	Spermatogonial stem cell
SSEA-4	Stage-specific embryonic antigen 4
StAR, STAR, Star	Steroidogenic acute regulatory protein, protein and gene names (human and rat)
Sult1e1	Hepatic sulfotransferase 1E1
SYCP3	Synaptonemal complex protein 3, gene name (human)
T3	Triiodothyronine
TDGF1	Teratocarcinoma derived growth factor 1, gene name (human)
TGFβ	Transforming growth factor beta
TLDA	Taq Man low density array
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor alpha
TPC	Testicular peritubular cells
Tra1-60	Tumor related antigen 1-60
VDAC-1	Voltage-dependent anion channel 1
WT1	Wilms tumor 1

1 INTRODUCTION

Infertility and its treatment is the primary concern in modern reproductive medicine. It is a growing problem affecting around 10 % of Western couples. The male factor with impaired sperm production or function accounts for half of the cases (WHO, 2008).
The arrival of stem cell technology has opened more possibilities to the field of regenerative medicine, and new strategies to approach male infertility have emerged.
A better understanding of key testicular cell types and their origin is crucial for the development of novel therapeutic approaches to testicular malfunction.

Leydig cells (LC) are the major source of steroids in the testis and important for the regulation of spermatogenesis and male development. The elucidation of paracrine factors and corresponding signaling pathways that control the differentiation process of stem Leydig cells into the adult Leydig cell lineage might open possibilities for pharmacological and dietary approaches to treat men with Leydig cell dysfunction or age-related decline in Leydig cell function.

Recent findings have shown that peritubular myoid cells (PTC) may contain putative stem Leydig cells (PSLC) [1]. The potential stem cell activity of PTCs could be used to restore spermatogenesis and testicular development. Adult PTCs could also serve as a source of somatic stem cells and may have potential in regenerative medicine. The identification of mechanisms underlying gonadal cell differentiation and the subsequent use of pluripotent stem cells in restoring spermatogenesis/ steroidogenesis is an important approach towards re-establishing fertility in a subpopulation of infertile or sub-fertile males. For example, male survivors of childhood cancer that have undergone gonadotoxic treatment are an increasing group of these patients.

1.1 INFERTILITY

Infertility is defined as the inability to conceive children after one year of frequent unprotected intercourse [2]. It has become a growing problem in the Western world concerning 10-15% of all couples (WHO) [3].

In about 50% of the infertility cases the male factor is involved and predominates in about 20%. The female factor is responsible for about 38% of the cases and the remaining 12% have no clear diagnosis and are therefore registered as idiopathic infertility [4].

Male fertility depends on complete spermatogenesis with normal hormonal levels, functional epididymal maturation and normal storage and transport of sperm [4].

Possible causes for male infertility include the dysfunction of the hypothalamic-pituitary gonadal axis (*e.g.* hypothalamic/ pituitary diseases that induce secondary hypogonadism) and the male genitalia (*e.g.* testicular diseases like primary spermatogenesis failure, anatomical abnormalities or primary hypogonadism and posttesticular defects generating obstructive azoospermia) [5].

Infertility can also result from cancer or other medical treatment, genetic disorders, sexually transmitted diseases, congenital malformations, surgery of the male genitalia or the inguinal region or may be caused by psychosexual problems [4].

1.2 THE TESTIS

The testis is one of the male genital organs. Its main functions are the production and maturation of sperm (spermatogenesis), as well as the production and secretion of androgens (steroidogenesis).

The testicular function is regulated by the hypothalamus and the pituitary, as well as by paracrine and autocrine factors, which are involved in both the spermato- and steroidogenesis.

For optimal function, the testes are located in the scrotum with organ temperature slightly below the body core temperature (human: 37˚C). The adult human testis is approximately 4-5 cm long, weighs 20 g and has a volume of 12-30 ml [6].

Histologically, the organ consists of about 600, closely packed seminiferous tubules, which correlate to about 360 m of seminiferous epithelium [2].

1.2.1 Histology

Due to its tubular structure, the organ can be divided into a tubular and an interstitial compartment, which are separated from each other by a basal lamina (Figure 1). The integrity of both compartments is necessary for the quantitatively and qualitatively normal sperm production.

The peritubular cells surrounding the tubuli seminiferi, the Sertoli and the germ cells build the tubular compartment, where the spermatogenesis takes place. The tubular compartment accounts for about 60-80% of the total testicular volume [2].

The interstitial compartment consists of Leydig cells, which represent 10-20% of the interstitial volume [2], macrophages and other immune-competent cells, vascular smooth muscle cells and pericytes.

In humans, the interstitium corresponds to about 12-15% of the total testicular volume [2].

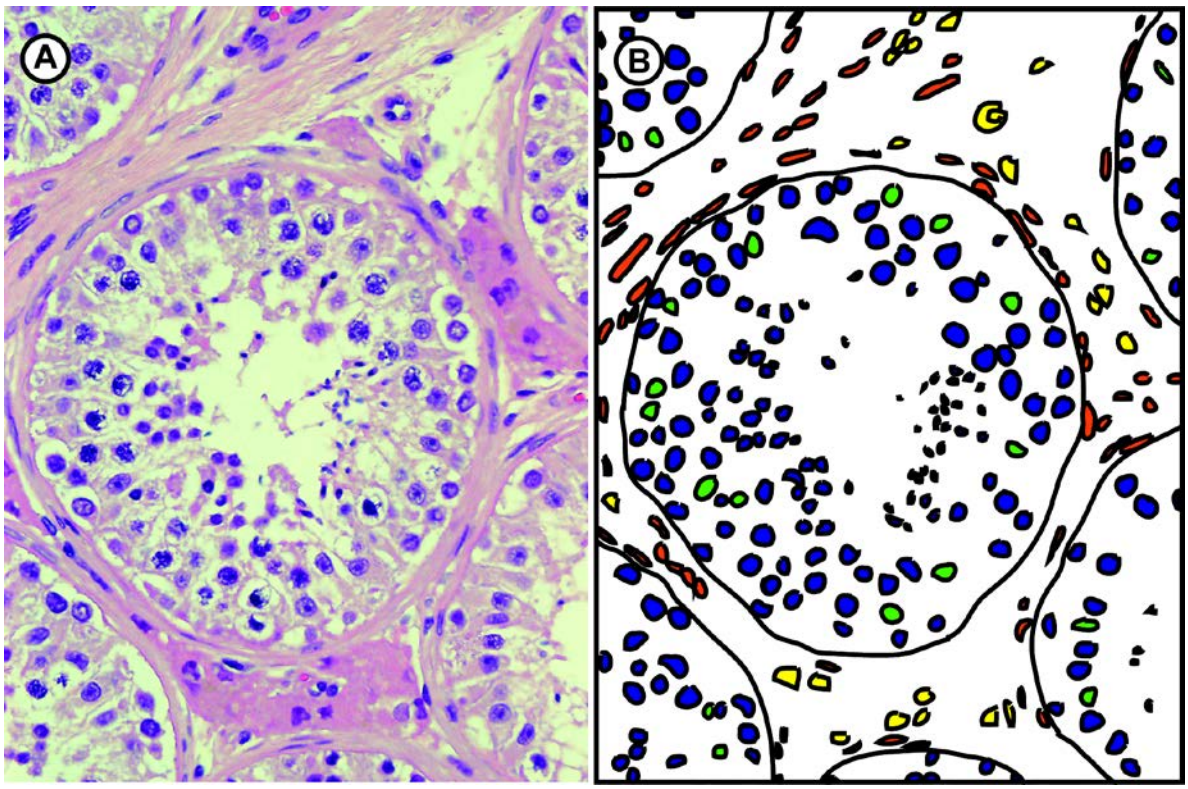

Figure 1: A: Testicular section of a healthy 23 year old man (Picture provided by J.-B. Stukenborg); B: Overview of the same testicular section with color coded cell nuclei: orange: peritubular cells, yellow: Leydig cells, green: Sertoli cells, blue: germ cells

1.2.1.1 Peritubular cells

The peritubular cells are smooth-muscle-like cells that build the wall of the tubuli seminiferi and are involved in sperm transport by tubular contractions. Together with the Sertoli cells, they are part of the spermatogonial stem cell niche and secret factors that orchestrate testicular functions. An in-depth description of the peritubular cells follows in chapter 1.4.

1.2.1.2 Sertoli cells

The Sertoli cells were first described in 1865 by Enrico Sertoli. They account for 35-40% of the volume of the germinal epithelium [2] and are the supporting structure of the germinal epithelium.

Their main function is to nourish and support the germ cells during differentiation. Complete spermatogenesis depends on Sertoli cells, as they are in a constant and intimate interaction with germ cells during all stages of their differentiation [7].

During fetal development, the Sertoli cells secrete factors such as Dhh and PDGF and thereby support fetal Leydig cell development [8]. In the prepubertal testis, their role is to maintain the peritubular phenotype and they are involved in the differentiation of the adult Leydig cell lineage [9].

The tight junctions of the Sertoli cells build the blood-testis barrier, which is formed when the first germ cells undergo meiotic division. The blood-testis barrier is part of the testicular defense mechanisms and separates the meiotic germ cells from the circulating blood.

The Sertoli cell function is hormonally regulated. Sertoli cells express FSH- and androgen receptors and can aromatize testosterone to estrogen. They secrete several proteins such as AMH, Sry, Sox9, transferrin and the androgen-binding protein, which are specific for Sertoli cells and can be used as markers for cell identification.

A healthy human testis with complete spermatogenesis contains 800-1200*10^6 Sertoli cells [2]. The relation between Sertoli and germ cells has been determined at 10:1.5 germ cells/ spermatozoa per Sertoli cell [2]. The total number of Sertoli cells regulates the final testicular size.

A gene knock-out in the murine Sertoli cell specific androgen receptor (AR) resulted in a normal male phenotype with reduced testicular size due to spermatogenetic arrest in meiosis [10, 11].

1.2.1.3 Germ cells

The germ cells are responsible for transfer of genetic information from one generation to the next. They differentiate in the tubular wall towards the lumen. Their development and function will be discussed in more detail in chapter 1.2.2.1.

1.2.1.4 Leydig cells

The Leydig cells are the steroid-producing cells of the testis. Their function is important for functional spermatogenesis and the well-being of the male. Steroidogenesis and the different Leydig cell types will be discussed in more details in the chapters below.

1.2.1.5 Macrophages and other immune-competent cells

The immune-competent cells play an important role in the regulation of testicular function. The testis is a unique immunoregulatory environment with limited immune response. While immune cells have unrestricted access to the interstitium, they cannot enter the tubular compartment because of the blood-testis-barrier.

Interestingly, the macrophage/ Leydig cell ratio differs between species. While rodents have about 1 macrophage per 4-5 LCs [12], in humans 1 macrophage corresponds to 10-50 Leydig cells [2].

Macrophages are involved in the regulation of Leydig cell development and function by secretion of several immunoregulatory factors (*e.g.* IL1β, IL1α, TNFα, LIF, IL2, IL6, TGFβ, prostaglandin and 25-OH-cholesterol) [13-15]. 25-OH-cholesterol bypasses StAR and supports basal steroidogenesis.

1.2.1.6 Vascular smooth muscle cells and pericytes

Vascular smooth muscle cells surround the blood vessels in the interstitium. Their role as putative precursors for the Leydig cell lineage has been discussed [16].

1.2.2 Testis Function

1.2.2.1 Spermatogenesis

Spermatogenesis refers to a series of differentiation steps leading from spermatogonia to mature spermatozoa and is divided into four phases, namely spermatogoniogenesis, forming of spermatids, spermiogenesis and spermiation (Figure 2).

Spermatogoniogenesis is the mitotic proliferation and differentiation of diploid germ cells (spermatogonia). The following meiotic division of double-diploid germ cells (spermatocytes) results in haploid germ cells called spermatids. This step involves two meiotic divisions: In meiosis I, a daughter cell with 23 pairs of chromosomes, the diploid primary spermatocyte is formed, in meiosis II, the cells containing diploid chromosomes are split into two haploid secondary spermatocytes with 23 single chromosomes.

During the following spermiogenesis, spermatids mature into spermatozoa. An acrosome cap is formed and the remaining cytoplasm is disposed in form of a residual body. The spermatozoa are then released from the germinal epithelium into the tubular lumen (spermiation) [2] and transported to the epididymidis for final maturation .

Spermatogenesis begins in puberty and is located in the tubuli seminiferi. The spermatogonial stem cells (SSCs) are located in a niche formed by the Sertoli and the peritubular cells. The differentiating cells migrate from the basal lamina towards the lumen of the seminiferous tubuli. During their passage, they are nourished and protected by the Sertoli cells. Once started, spermatogenesis occurs throughout the entire adult life cycle.

The entire process of spermatogenesis is hormonally regulated by testosterone and LH/FSH, and exhibits major interspecies differences between rodents and humans.

In rodents, spermatogenesis involves 12 stages: three undifferentiated spermatogonia types (*i.e.* A single, A paired, A aligned) and six differentiating spermatogonia (*i.e.* A1, A2, A3, A4, intermediate and B spermatogonia) giving rise to two daughter cells per division. The secondary spermatocytes, also called type B spermatogonia, further differentiate through meiosis into spermatozoa and finally into mature sperm. This results in 4096 elongated spermatids from one SSC in each cycle of spermatogenesis.

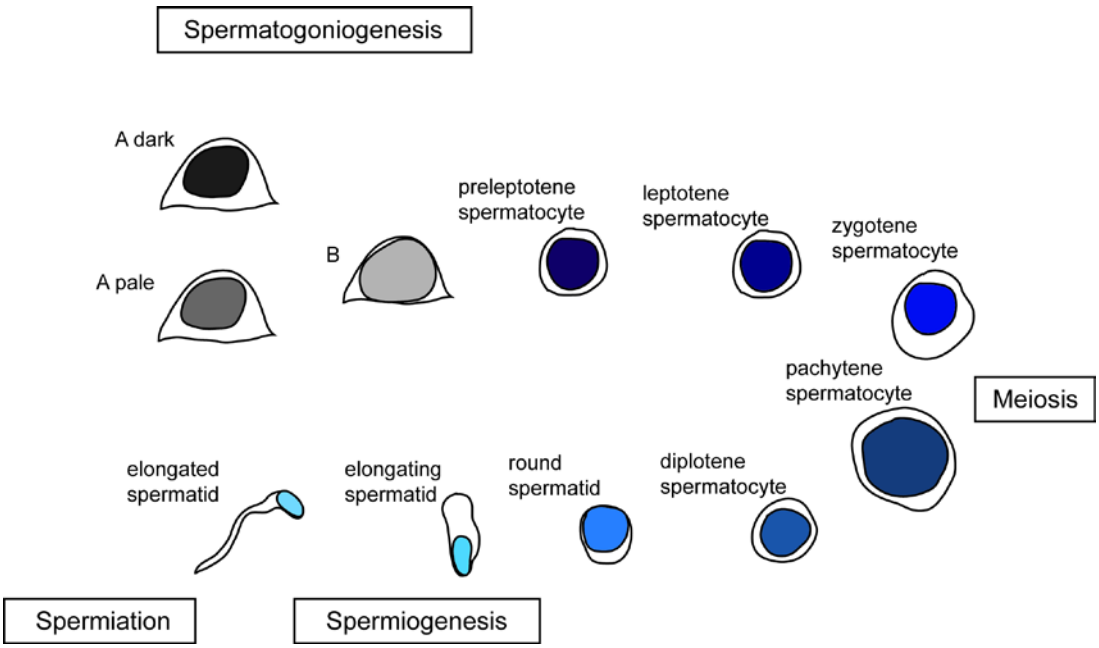

Figure 2: Schematic overview of the human spermatogenesis adapted from J-B. Stukenborg

In contrast, human spermatogenesis involves six stages and includes three different types of spermatogonia: A dark, the self-renewing stem cell, A pale and B spermatogonia [17]. The subsequent differentiation process of B spermatogonia is similar to the spermatogenesis in rodents [18, 19] (Figure 2).

Spermatogenesis takes 64 days with one spermatogenic cycle every 16 days [2].One A pale spermatogonium only gives rise 16 elongated spermatids in one cycle.

1.2.2.2 Steroidogenesis

Steroidogenesis describes the synthesis of steroids from cholesterol, with testosterone being the final product in adult Leydig cells. In addition to Leydig cells, steroidogenesis also occurs in the adrenals and in the theca cells of the ovary. There are several sources for the substrate cholesterol: *de novo* synthesis, from cholesteryl ester storage, supplied exogenously by lipoproteins or retrieved from the plasma membrane [20].

The two main organelles involved in steroidogenesis in Leydig cells are the mitochondria and the smooth endoplasmatic reticulum (SER). Cholesterol is transported to the mitochondrion for conversion to pregnenolone the side chain cleavage enzyme P450scc. Following the transport into the smooth endoplasmatic reticulum, several conversion steps, involving the enzymes 3βHSD, P450c17 and 17βHSD, convert pregnenolone into testosterone, which is finally released into the cytoplasm (Figure 3) [21, 22].

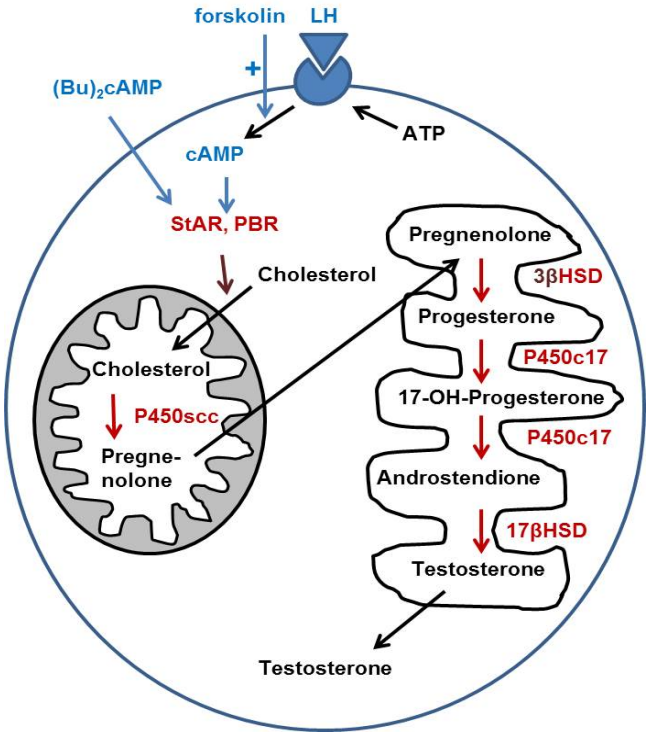

Figure 3: Overview of steroidogenesis in an adult Leydig cell. StAR and PBR transport cholesterol to the inner mitochondrial membrane and into the mitochondrion where the conversion of cholesterol to pregnenolone occurs. Subsequently, pregnenolone is transported to the smooth endoplasmatic reticulum where it is converted into testosterone which is then released into the cytoplasm prior to its release from the cell. Blue: stimulators of steroidogenesis, dark red: conversion steps and enzymes

Steroidogenesis is regulated both acutely and long term. StAR and a protein complex (transduceosome) including PBR (TSPO), VDAC-1 (voltage-dependent anion channel), PAP7 (TSPO-associated protein 7) and PKAR1A (protein kinase A regulatory subunit 1α) are involved in the acute regulation of steroidogenesis through control of cholesterol transport from the outer to the inner mitochondrial membrane [23-25]. Substrate availability is a rate-limiting step in steroidogenesis. Long-term regulation of steroidogenesis is facilitated via gene transcription changes involving LH stimulation [20].

Several pathways such as the cAMP-PKA pathway, the protein kinase c and the intracellular Ca^{2+} signaling are involved in the regulation of steroidogenesis [20].

1.2.2.3 Testosterone

Testosterone is the most important androgen in men. About 95% of the circulating androgens are synthesized in the testes, where the Leydig cells produces about 6-7mg per day [2]. Normal serum values are around 12-30 nmol/ L.

Androgens are responsible for the genesis of the male sexual characteristics. During embryonic development, the sexual organs are formed and their further development towards the male phenotype continues in puberty.

In the testis, several cell types express the androgen receptor, such as germ, Sertoli, Leydig, and peritubular cells. Androgens are involved in the regulation of meiotic and postmeiotic germ cell development and Sertoli cell proliferation, and control the maintenance of complete spermatogenesis, spermatocyte and spermatid development via the Sertoli cells [26]. In the Leydig cells, androgens regulate the testosterone production via a feedback loop and are required for normal male fertility [27]. Contractility of the peritubular cells, which ensures normal spermiation, is governed by androgens [28].

Testosterone has several other target organs in addition to the testis. It influences growth of all male sexual organs, i.e. penis, prostate, seminal vesicles and epididymidis. In the brain, it regulates cognitive function, socialization, dominance and libido. Furthermore, testosterone regulates both body growth and proportion and, it is involved in erythropoiesis, muscle and bone anabolism and lipid metabolism. It increases skin hair growth, balding and sebum production and it initiates growth of the larynx during puberty, resulting in a lower voice in males [2].

Testosterone deficiency leads to osteoporosis, pallor, wrinkled skin, anemia, muscle atrophy, psychological changes and loss of libido.

1.3 THE LEYDIG CELL

The Leydig cells are the testosterone producing cells in the testis and thereby the main testosterone producer in men. The most important LC markers are LHR, 3βHSD, INSL3. Adult human testes contain about $200*10^6$ Leydig cells [2], while the adult rat testes contain approximately $50*10^6$ Leydig cells [29].

LCs were first described in animals by Franz Leydig in 1850 [30] and in men by Albert Kölliker in 1854 [31], but their role remained unclear until testosterone was isolated for the first time in 1935 [32]. It took another 30 years until in 1965 the first biochemical evidence of this Leydig cell function was provided [33, 34].

From a developmental point of view Leydig cells can be divided into different subtypes. In the fetus, fetal Leydig cells (FLCs) produce testosterone and the INSL-3, which is needed for normal development of the male external genitalia and the testis descent into the scrotum. Androgen production declines after birth. In humans, there is a quiescent period during childhood. In puberty, the Leydig cell differentiation resumes and the mature adult Leydig cells are formed via progenitor and immature cells. They produce androgens during the entire adult life span [35].

To distinguish between the two Leydig cell generations, fetal and adult, respectively, Mc2r was described as a marker for fetal Leydig cells [36], and Sult1e1 for the adult Leydig cell lineage [37].

1.3.1 The fetal Leydig cell (FLC)

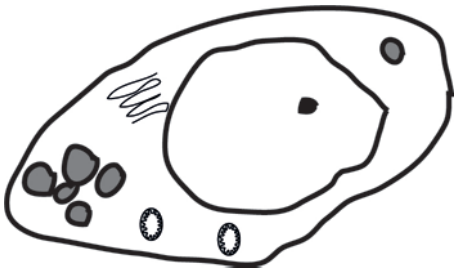

Figure 4: Schematic overview of a fetal Leydig cell with a big nucleus and nucleolus, lipid droplets (grey), smooth endoplasmatic reticulum and mitochondria

The origin of fetal Leydig cells has been a matter of debate for a long time. Evidence has been provided for their development from the adrenal-gonadal primordium [38], the neural crest [39, 40], the mesonephros [41, 42] or the coelomic epithelium [43, 44]. In rodents, they first appear around E12.5 (mice) and E14.5 (rat), whereas in humans, they are found from week 6-7 of pregnancy, immediately following the formation of the testis cords.

FLCs differentiate in the interstitium into steroidogenic cells. They are of oval shape and possess abundant smooth endoplasmatic reticulum, mitochondria and lipid droplets (Figure 4). While in the interstitium, they form clusters that are surrounded by a basal lamina. Their formation is dependent on Dhh and PDGF-A, two factors produced by Sertoli cells [8, 45], but independent of LH because LHR expression is not found until after the onset of FLC development. Furthermore, a LHR-knock out mouse shows unchanged testosterone levels during the fetal period [46].

FLCs are competent to produce testosterone at E 15.5 in rats [29, 47] and from week 6-7 in humans [45]. Testosterone controls the development of the male characteristics by formation of the internal and the external genitalia as well as influencing neuroendocrine functions. Testosterone production peaks in week 14-18. The cells continue to proliferate and reach their maximal number in week 15 (rat E19.5) and during week 18-38 they start to involute. Around birth about $18*10^6$ cells are left, which decrease in volume [48] and remain quiescent until puberty.

There is a second wave of testosterone production after birth, also known as mini puberty, taking place at month 2-3 [49, 50].

In addition, FLCs produce Insulin-like 3 (Insl3), a growth factor that is important for the abdominal phase of the testicular descent- through stimulation of the growth of the gubernaculum [51]- and as a survival factor for germ cells.

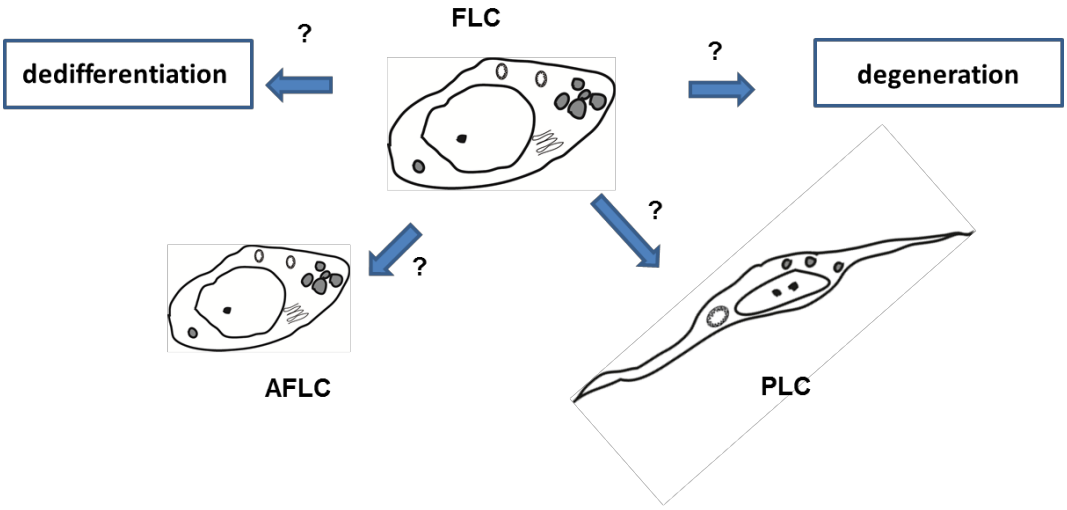

Figure 5: Schematic overview of possible fate of FLCs after birth; FLC: fetal Leydig cell, AFLC: adult fetal Leydig cell, PLC: progenitor Leydig cell

There are several theories about the developmental fate of the FLCs. It was suggested that they undergo degeneration [48], dedifferentiate towards fibroblastic cells [52], persist as a second Leydig cell type along with ALCs [53, 54] or re-differentiate and give rise to the adult Leydig cell lineage [55]. Figure 5 gives a tentative model of the FLC fate.

1.3.2 Development of the adult Leydig cell lineage

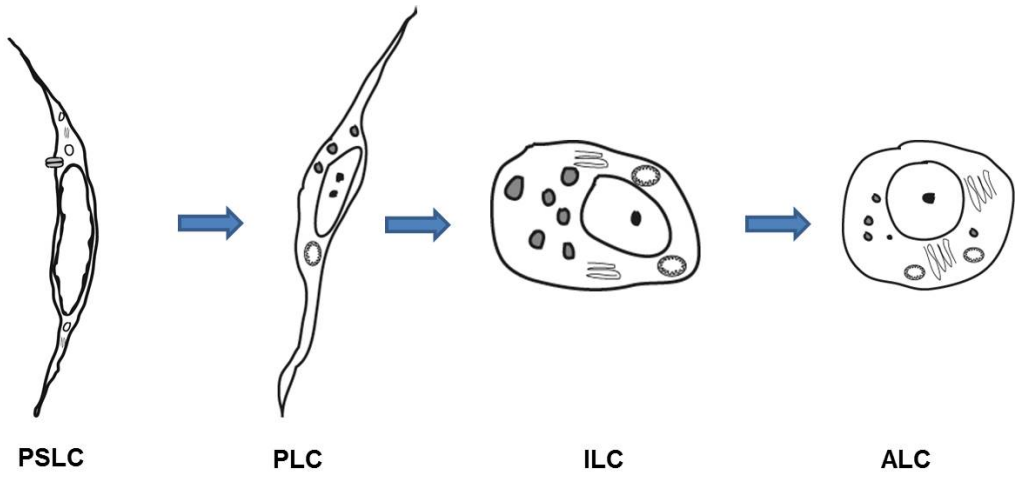

Figure 6: Leydig cell development from putative stem Leydig cell (PSLC) to progenitor (PLC), immature (ILC) and mature adult Leydig cell (ALC)

The development of the adult Leydig cell lineage begins in rodents after birth and in humans after the quiescent childhood period with the onset of puberty. Putative stem

Leydig cells (PSLCs) give rise to a progenitor cell type (PLCs) that further differentiates into immature Leydig cells (ILCs) followed by their maturation into adult Leydig cells (ALCs) (Figure 6). Testosterone production by mature LCs is required for the onset of spermatogenesis [29].

Several factors have been described to be involved in differentiation (*e.g.* LH, Dhh, PDGF-A [56] and cAMP) and in proliferation (*e.g.* LIF, PDGF, LH [56] and cAMP) of ALCs.

In contrast to fetal Leydig cells, adult Leydig cell differentiation is LH dependent. In LHR-knock out mice the total number of Leydig cells is reduced and androgens levels are undetectable [46].

The adult Leydig cells are responsible for androgen production during the adult lifespan. However, aging causes testosterone levels to decline about 50% [29].

1.3.3 Stem Leydig cell (SLC)

Figure 7: Schematic overview of a putative stem Leydig cell; a mesenchymal-like cell with slender cytoplasm and elongated nucleus, long cytoplasmatic processes; grey receptor: PDGFRα

Stem Leydig cells have a mesenchymal-like phenotype [12]. Morphological studies have shown that peritubular mesenchymal cells located in the outermost line of the peritubular compartment can give rise to the adult Leydig cell lineage [12, 55, 57, 58].

At the same time, vascular smooth muscle cells and pericytes of testicular blood vessels have also been proposed as possible precursor of ALCs [16], resulting in the hypothesis of a multifocal origin of Leydig cell precursors in neonatal testis [49, 59, 60].

At day 7, spindle-shaped LHR- and 3βHSD-negative cells are found in the peritubular region of the neonatal rat testis (Figure 7). These cells express PDGFRα, LIFR and c-kit [1]. After their enrichment via PDGFRα their capacity for self-renewal, differentiation *in vitro* and replenishment of the peritubular niche *in vivo* has been demonstrated [1].

The PDGFRα has been shown to be expressed at all stages of Leydig cell development with a peak in the low differentiated progenitor Leydig cell population [61] and in neonatal peritubular cells [62].

PDGF-A-deficient mice do not develop the adult Leydig cell lineage [63], suggesting that PDGFRα plays an important role in the development of these testicular cells.

1.3.4 Progenitor Leydig cell (PLC)

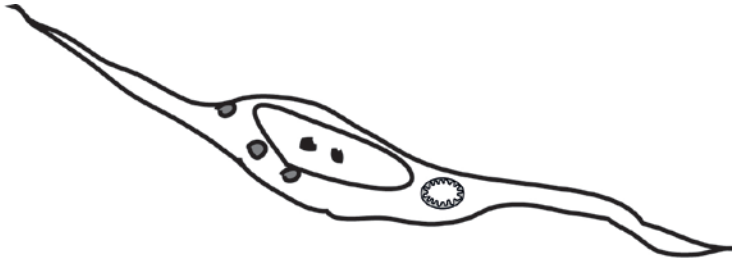

Figure 8: Schematic overview of a progenitor Leydig cell; showing the typical spindle shape, the nucleus with nucleoli, some lipid droplets (grey) and a mitochondrium

Prior to day 10 in rats, the stem Leydig cells are committed to the Leydig cell lineage and differentiate towards progenitor cells. These mesenchymal-like fibroblasts [12, 59] are found in the outer layer of the seminiferous tubules and perivascular in the rat testis between day 11-28 and before three years of age in humans.

PLCs still resemble the elongated and spindle-shape of stem cells, but they contain mitochondria and lipid droplets that are typical organelles of steroid producing cells (Figure 8). PDGFRα, LIFR and c-kit expression is still present [35], but in contrast to the stem cells, PLCs also express the steroidogenic enzymes P450scc, 3 βHSD, P450c17, 3αHSD, 17βHSD, as well as a truncated form of LHR and AR [12, 59, 64].

They produce androsterone as their endpoint steroid [65]. Progenitor Leydig cells are highly proliferative with a peak quantity at day 13 [66].

1.3.5 Immature Leydig cell (ILC)

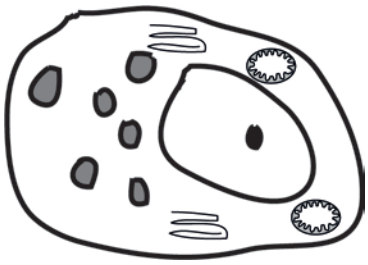

Figure 9: Schematic overview of an immature Leydig cell; the shape is rounder, the cell contains a nucleus with nucleolus, numerous lipid droplets, smooth endoplasmatic reticulum and mitochondria

From day 28-56 in rats, and between 3-8 years in humans [12, 67], immature Leydig cells are the most prominent Leydig cell type in the testis. The phenotype of these cells is rounder and they contain numerous lipid droplets (Figure 9).

ILCs express high levels of testosterone metabolizing enzymes (*e.g.* 5α reductase II and 3αHSD) that convert testosterone into its metabolites, with 5α-androstane-3α,17β-diol as the main steroid [35].

The ILCs move from the peritubular and perivascular region towards the interstitium [57], where they further mature into adult Leydig cells.

1.3.6 Adult Leydig cell (ALC)

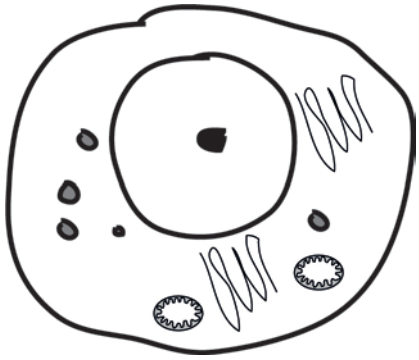

Figure 10: Schematic overview of an adult Leydig cell; the round cell contains a nucleus with nucleolus, fewer lipid droplets, smooth endoplasmatic reticulum and mitochondria

From 9-11 years in humans, ILCs start to mature [68] and with 15 years (day 56 in rats) [69], the maturation of the Leydig cell lineage has come to an end, making mature adult Leydig cells the dominant Leydig cell in the testis.

Fully differentiated adult Leydig cells have a very limited capacity to proliferate [70]. However, cell regeneration after elimination with ethane-1,2-dimethyl sulphonate (EDS) is possible [71].

ALCs are round in shape and contain smooth endoplasmatic reticulum (SER), few lipid droplets and mitochondria (Figure 10).

In humans, subunits of globular proteins with an unknown function called Reinke crystals are found in adult Leydig cells [35, 68].

ALCs express all steroidogenic enzymes. They produce testosterone as the main steroid and Insl3. Classical markers for LC identification are LHR and Insl3.

1.4 PERITUBULAR CELL (PTC, TPC)

Figure 11: Schematic overview of a peritubular cell; slender cytoplasm with elongated nucleus, long cytoplasmatic processes

Peritubular cells are smooth muscular cells surrounding the seminiferous tubule (Figure 11). In rodents, PTCs form a single layer around the tubules, whereas several cell layers with different phenotypes are found in humans [72]. Cells of the inner layer have more muscular characteristics and express desmin as typical marker. Towards the outer layer PTCs increasingly express vimentin and resemble more and more connective tissue cells [73].

For a long time, the endothelin-controlled tubule contraction- for sperm transport to the rete testis- was considered to be the most important function of PTCs [28, 74-76]. However, recently, it has become evident that PTCs play additional roles in male fertility [77].

As Sertoli cells, they secrete paracrine factors and ECM proteins [72, 78-81] and contribute to the spermatogonial stem cell niche [72, 80, 82].

The secretome of PTCs has been shown to contain fibronectin, collagen1, collagen 4, laminin, nexin and cystein [82, 83].

Classical markers for PTCs are α-SMA [84, 85] and Myh11 [79] (targeting their muscular characteristics), CD90/Thy-1 (their fibroblast property), [83] and alkaline phosphatase [86].

Testicular size is determined by PTC proliferation in the longitudinal growth of seminiferous tubules [77]. Their proliferation coincides with that of Sertoli cells and ceases before puberty. Sertoli cells help to maintain the PTC phenotype during prepubertal development [9]. During puberty, the peritubular cells mature, and cytoarchitectural changes such as flattening of the cells lead to an increase in the peritubular surface [87].

Already during fetal life, peritubular cells express AR, which is used by androgens to induce smooth muscle characteristics [88]. PTC- specific AR depletion causes infertility and a reduced testicular size in mice [76].

Besides AR, PTCs also express PDGFRα and PDGFRβ [62, 81]. The PDGF signaling is involved in the regulation of the contractility. Stimulation of PTCs with PDGF causes their hypertrophy [89, 90].

Expression of PDGFRα, an established stem Leydig cell marker [1]-, and morphological studies suggest that PTCs could be precursors for the ALC lineage [12, 57, 91].

In addition, PTCs express functional receptors for histamine and tryptase [83] and are activated by mast cells. In infertile men, phenotypic changes of the PTCs have been described. In these cases, PTCs produced higher amounts of ECM components, causing fibrosis [72, 83].

1.5 PLATELET-DERIVED GROWTH FACTOR RECEPTOR ALPHA (PDGFRA)

The PDGF receptor α has been described as an important marker for the adult Leydig cell lineage [1, 92] and has subsequently been used to isolate stem Leydig cells from prepubertal rat testes [1].

PDGF receptors play a well-characterized role in proliferation and migration of mesenchymal cell types [8]. While the PDGFRβ solely binds to PDGF-B, the PDGFRα has a broader spectrum and binds PDGF-A, PDGF-B and PDGF-C as well as AB heterodimers [93-95].

PTCs and Leydig cells at all developmental stages express the PDGFRα [1, 61, 62, 92]. While PDGF-A-deficient mice lack the adult Leydig cell lineage [63], a PDGFRα-knock-out leads to a smaller number of fetal LCs [8]. Taken together, these findings suggest that the PDGF signaling plays an important role in LC development.

1.6 CAMP-PKA-PATHWAY

The cAMP-PKA pathway plays an important role for Leydig cell function and is involved in the regulation of steroidogenesis (Figure 3), metabolism, gene activity, cell growth and division, differentiation, sperm motility as well as ion channel conductivity [96].

The cAMP-PKA pathway is a G protein-coupled receptor (GPCR)-triggered signaling cascade. Upon stimulation the activated G_s alpha subunit binds to and triggers the enzyme adenylyl cyclase, which converts ATP into cyclic adenosine monophosphate (cAMP). The second-messenger cAMP stimulates PKA, which, in turn, phosphorylates transcription factors and thereby regulates protein expression.

LH, FSH and ACTH signal through GPCRs to activate steroidogenesis. These signals up-regulate StAR expression and activate the cholesterol ester hydrolase, which increases intracellular cholesterol levels [97, 98]. Furthermore, the cAMP-PKA signaling is involved in the up-regulation of mRNAs encoding steroidogenic enzymes [99, 100].

To simulate the adenylate cyclase activation through PKA signaling, cells can be treated with exogenous cAMP. $(Bu)_2$cAMP, a metabolite of cAMP with two additional butyryl groups, can bypass the LHR, and raise intracellular cAMP levels in cells without a functional LHR (Figure 3).

Forskolin activates the adenylate cyclase and thereby enhances cAMP production (Figure 3).

1.7 HUMAN EMBRYONIC STEM CELL (HESC)

1.7.1 Embryogenesis

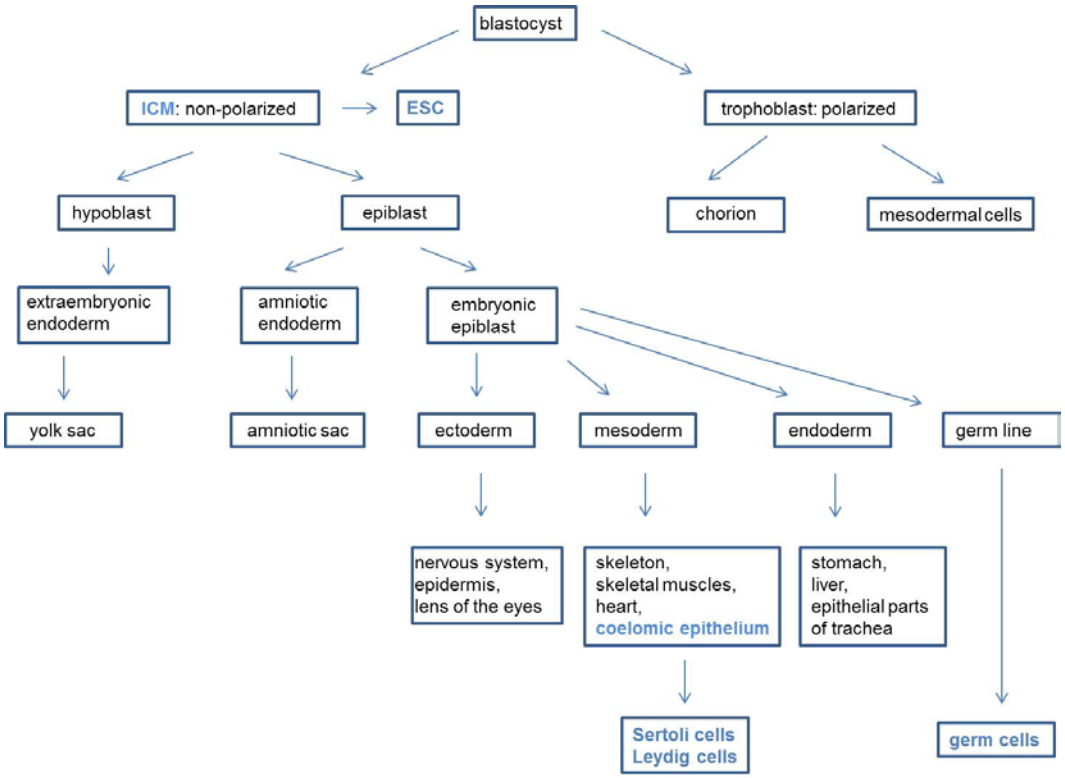

Figure 12: Schematic overview of embryogenesis; cells and tissues mentioned in more detail in the thesis are marked in blue

Embryogenesis describes the development of the embryo until gastrulation [101]. It begins with the formation of a zygote when the spermatozoon and the oocyte combine.

In the zygote, the cells undergo mitotic divisions. When the eight cell stage is reached, the zygote is called blastomere and develops further into the morula at the 16-cell stage. The morula is divided into two layers, an inner non-polarized and an outer polarized layer.

Subsequently, the blastocyst formation starts, and a fluid-filled cavity called blastocoel develops. In humans, this stage is reached five days post fertilization.

The two layers of the blastocyst develop further into the inner cell mass (ICM), formed from the non-polarized cells, and the trophoblast layer, formed from the polarized cells (Figure 12).

The ICM then gives rise to extra-embryonic membranes and the embryo, while the trophoblast layer differentiates into the embryonic part of the placenta (chorion) and the mesodermal cells.

The placenta consists of the chorion and the maternal part, the decidua. During pregnancy, the placenta is used as nutrition source and waste disposal. Furthermore, it plays an important role as an immune-rejection block.

The blastocyst enters the uterus and starts to produce plasmin, which degrades the zona pelucida and thereby facilitates the hatching of the blastocyst into the uterus wall. At that point, the ICM has formed a bilaminar disc containing a hypo- and an epiblast.

The hypoblast develops further into the extra-embryonic endoderm and forms the yolk sac, while the epiblast differentiates into the amniotic endoderm and the embryonic epiblast.

The amniotic endoderm builds the amniotic sac, which is filled with amniotic fluid and contains the embryo.

The embryo is formed out of the embryonic epiblast that differentiates into the three germ layers and germline cells [102]. During gastrulation, the primitive streak and the three germ layers emerge. The germ layers will give rise to the differentiated organs. The mesoderm forms *e.g.* the skeleton, the skeletal muscles, the heart, the coelomic epithelium and the gonad somatic cells as well as the testicular interstitial cells. The ectoderm further differentiates into *e.g.* the nervous system, the epidermis and the lens of the eyes. The endoderm will develop into *e.g*. the stomach, the liver and the epithelial parts of the trachea.

Primordial germ cells are the germ cell precursors that transfer the genetic information from one generation to the next. They migrate from the yolk sac wall into the genital ridge to further differentiate into gonocytes. In humans, this happens 4-6 weeks after gestation [102].

While in females gonocytes become meiotic oocytes, in males gonocytes undergo mitotic arrest and become quiescent prespermatogonia that restore meiosis after birth.

1.7.2 Embryonic stem cell (ESC)

Human embryonic stem (hES) cells are of great potential use in regenerative medicine. They serve as an important tool in human disease modelling, drug discovery [103] and the study of differentiation pathways.

The first mouse ES cells were isolated in 1981 from 4-5 day old mouse embryos by Evans and Kaufman [104]. Several years later in 1984, Fishel and Edwards achieved the first isolation of hES cells [105], but not until 1994 the first hES cell line was cultured by Bongso [106].

hES cells are derived from the inner cell mass of a human blastocyst at day 5-8 after fertilization [107, 108] or from 8-cell stage embryos [109].

These pluripotent cells have the potential to differentiate into all three germ layers and germ line cells *in vivo* as well as *in vitro*. ES cells have the capacity for self-renewal and express pluripotency markers (*e.g.* Pou5f1, Nanog, the stage specific embryonic antigen [SSEA4], the tumor related antigen [Tra1-60] and Sox-2). After transplantation into immunodeficient (SCID) mice, they form teratomas.

In serum free conditions, self-renewal depends on FGF2 [110], whereas differentiation is induced BMP stimulation [111].

To date standard culture conditions for hES cells require fetal mouse or human foreskin fibroblasts [107, 112], but defined xeno- and feeder-free systems are needed for hES cells to avoid problems with immunogenicity, microbial or viral contamination, and variability [113].

1.7.3 Laminin (LN)

The classical feeder-free culturing system uses matrigel, a protein mixture derived from mouse Engelbreth-Holm-Swarm tumors [103] that contains LN111, type IV collagen, perlecan and nidogen, growth factors and several unknown components [113]. The batch-to-batch variability creates problems for the comparability between hES cell experiments.

To use ECM proteins as matrix instead of matrigel has several advantages. These proteins are part of *in vivo* niches of stem cells and facilitate differentiation, phenotype maintenance, adhesion, function of many cell types, proliferation, migration and self-renewal [113].

Laminin is an important component of basement membranes [113] and the most dominant component of matrigel [103]. It binds to receptors on cell surfaces, *e.g.* integrins, which are major adhesion receptors of hES cells [114].

Laminins build a family of heterotrimeric glycoproteins, containing α, β and γ chains. For example laminin 521, used in this work, consists of an α5, β2 and γ1 chain [115]. It is expressed by the ICM and hES cells, in hair follicles and in intestinal villus crypts [115].

On laminin, hES cells grow as a monolayer. The cells acquire a migration potential without differentiation that is beneficial for hES cell culturing. A second advantage of the monolayer formation is the increased cell accessibility, which facilitates a more

homogenous distribution of differentiation factors [113] and enhances the differentiation process.

1.7.4 Steroidogenic Factor 1 (SF-1)

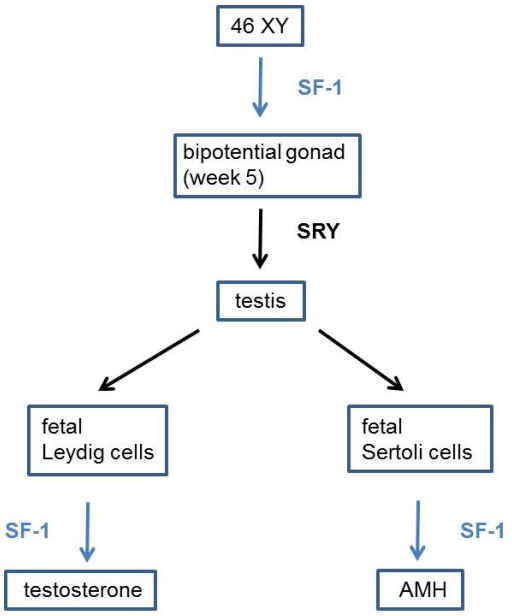

Figure 13: Role of SF-1 during male sexual differentiation, SF-1 function shown in blue

The steroidogenic factor 1 is involved in male sexual differentiation by determination of fetal Leydig and Sertoli cell function (Figure 13).

In addition, it has been described to facilitate steroidogenic cell differentiation of pluripotent cells [116-118].

SF-1 belongs to the nuclear hormone receptor family (NR5A), a group of gene-specific transcription factors involved in the formation of steroid hormones, thyroid hormone, vitamin D and retinoids [119].

SF-1 is a cell-selective orphan nuclear receptor and a key regulator of endocrine function in hypothalamic-pituitary-gonadal axis and sex differentiation [119].

It is expressed in the urogenital ridge, all three layers of the adrenal cortex, Leydig and Sertoli cells, ovarian granulosa and theca cells, the placenta, the pituitary and the hypothalamus [120].

Monomeric SF-1 binds to the responsive element located in promoter regions of different steroidogenic enzymes and enhances their transcription [119]. SF-1 is involved in the regulation of the expression of the steroidogenic enzymes P450scc, P450c17, 3βHSD and aromatase [119, 120] in the regulation of StAR, LHR, AMH

and SRY [121, 122]. The cAMP-PKA pathway can enhance SF-1 transactivation activity, on target genes such as CYP11A1 and STAR [123].

SF-1 deficiency leads to a lack of adrenal glands and gonads, a male-to-female sex reversal of internal and external genitalia, and death due to adrenocortical insufficiency [124].

The Liver Receptor Homologue 1 (LRH-1) is closely related to SF-1. However, it is encoded by a different gene and also expressed in the liver and pancreas [119, 125].

Both, SF-1 and LRH-1, regulate Oct3/4 expression and can therefore induce pluripotency in somatic cells [126-128].

1.7.5 *In vitro* derivation of steroidogenic cells

To learn more about early differentiation pathways involved in the development of Leydig cells, several groups have used *in vitro* derivation of steroidogenic cells from mouse and human embryonic and mesenchymal stem cells.

So far, only overexpression of SF-1 or LRH-1, respectively, has led to steroidogenic cell differentiation through alteration of chromosomal structures [116-118, 127, 129-132].

Crawford *et al.* ectopically expressed SF-1 in mES cells and found progesterone producing cells upon the addition of 20α-hydroxycholesterol, a substrate for steroidogenesis that is able to bypass the outer mitochondrial membrane. The derived cells were shown to express endogenous CYP11A1, and the expression was increased through cAMP/RA treatment [116].

Gondo *et al*. transfected mouse bone marrow and mesenchymal stem cells derived from adipose tissue with SF-1 and described the development of ACTH responsive cells that expressed genes for steroidogenic enzymes and produced testosterone. They found that adipose derived cells were more similar to adrenal cells, while the bone marrow derived cells showed a more gonadal phenotype [117, 130].

Also Jadhav *et al.* has worked with murine cells. They transfected mES cells to overexpress SF-1 and obtained progesterone secreting cells. LIF removal for 7d followed by cAMP stimulation for 4d further differentiated these cells towards testosterone and estradiol production as well as *de novo* cholesterol synthesis.

Most studies involving material of human origin use bone marrow derived mesenchymal cells. Tanaka *et al.* overexpressed SF-1 in human bone marrow cells and detected ACTHR and LHR expression as well as testosterone production in the differentiated cells [118].

As a complementary approach, overexpression of LRH1, another member of the nuclear hormone receptor family (NR5A), was compared to SF-1 by Yazawa *et al*. Overexpression of both LRH-1 and SF-1 in human MSCs followed by cAMP

stimulation led to steroidogenic enzyme expression and steroid production in the cells. To be able to differentiate hES cells into steroidogenic cells, the cells were first pre-differentiated towards the mesenchymal cell line before overexpression of SF-1 induced steroidogenic cell formation [131, 133, 134].

Sonoyama *et al.* were able to show that SF-1 transfection followed by treatment with cAMP for 7-14 days differentiated hES and hIPS cells into adrenocortical cells, while differentiation of hES cells through embryoid body formation resulted in trophoblast like cells [129].

2 PROJECT AIMS

The general aim of this thesis was to gain more knowledge about the development of Leydig cells. The phenotype and the possible relationship of rat and human peritubular cells with the adult Leydig cell lineage were analyzed. Furthermore, the potential of human embryonic stem cells to differentiate into steroid-producing cells was evaluated.

The specific aims of the four projects were:

1) To analyze the phenotype, steroidogenesis and proliferative activity of postnatal fetal Leydig cells.

2) To characterize PDGFRα-positive peritubular cells in neonatal rat testes and to explore their potential to differentiate into the adult Leydig cell lineage.

3) To describe PDGFRα-positive human testicular peritubular cells and to examine their potential for differentiation into the adult Leydig cell lineage.

4) To screen different hES cell lines for their differentiation potential towards gonadal cell types.

3 METHODOLOGY

3.1 ETHICS

All studies were performed with permission from an Ethical Board. For Project 1 and 2, “Stockholms Norra Djurförsöksetiska Nämnd” approved the animal experiments (N319/08, N143/08, N305/09, N489/11). Project 3 was performed under the ethical permission of the “Ethikkomission der Fakultät für Medizin der Technischen Universität München” (3051/11) and Project 4 was authorized through the “Forskningsetikkommitté Syd” (454/02).

All efforts were taken to minimize suffering of animals and patients.

3.2 CELL CULTURE

Cultivation of freshly isolated cells and cell lines was a key part of all projects in this thesis.

In Project 1 and 2, cells were freshly isolated from rat testicular tissue and kept in culture for up to one week. All cell cultures are to some degree artificial systems that affect cell behavior through the *in vitro* culture conditions, but primary cell cultures resemble the *in vivo* situation more closely than cell lines. However, the need of a constant animal supply for their creation is an important draw back and experimental reproducibility relies heavily on the repeatable recreation of cell cultures derived from different animals.

In Project 3, cells were derived from human testicular biopsies but kept in culture for several passages and are therefore more similar to cell lines. In Project 4, seven human embryonic cell lines were used.

From an ethical point of view, the use of cell lines is preferable. Higher reliability and reproducibility of the experiments are clear advantages, but the artificial culture conditions may change cell behavior substantially and limit the transferability of the results to the *in vivo* situation.

3.2.1 Rat fetal Leydig cells and peritubular cells (Paper I & II)

Postnatal fetal Leydig cells and peritubular cells were isolated from 7-8 day old male SD-rat pups housed under standard conditions in the animal facility of Karolinska Institutet.

After decapitation of the pups, the testes were decised and decapsulated. The tissue was mechanically and enzymatically disintegrated and the interstitial cells were separated by filtration and centrifugation.

To isolate the FLCs, the cell preparation was incubated with an antibody against LHR, an established Leydig cell marker, and a magnetic cell separation technique (MACS) was used for purification.

In Project 2, testicular cell suspensions depleted of fetal Leydig cells were used to isolate PDGFRα-peritubular cells using the MACS technology. The isolated cells were LHR-negative and PDGFRα-positive. This expression profile has previously been described for putative stem Leydig cells [1].

In both projects, the isolated cells were transferred into DMEM+F12 containing 2% FCS and cultured at 34˚C and 5% CO_2. After stimulation with different factors like $(Bu)_2$cAMP, hCG, PDGF-A and LIF for up to 72 hours the cells were analyzed for their proliferation- and differentiation-potential as well as their capacity for steroid production *in vitro*.

3.2.2 Human testicular peritubular cells (Paper III)

Human testicular peritubular cells (HTPCs) that were used in Project 3 were isolated by outgrow culture from testicular biopsies from patients with obstructive and non-obstructive azoospermia. Biopsies were routinely taken prior to relegation of the vas deferens or intracytoplasmatic sperm injection treatment.

Patients suffering from obstructive azoospermia have a post testicular blockage of the sperm delivery but normal spermatogenesis, while non-obstructive azoospermia is caused by testicular sperm production failure.

After isolation, the HTPCs were cultured in DMEM with phenol red, a high concentration of glucose (4.5g/l), L-glutamine and 10% FCS for 5-10 passages prior to experiments.

To stimulate proliferation, differentiation and steroid production, the cells were treated with forskolin or PDGF-B for 6 to 24 hours.

3.2.3 Human embryonic stem cells (Paper IV)

Human embryonic stem cells (hESCs) originate from the inner cell mass of a human blastocyst at 5-8 days after fertilization. All seven cell lines were derived from the group of Prof. Outi Hovatta at the Karolinska Institutet in Huddinge.

Before the start of the project, hES cells were cultured on human foreskin fibroblasts for 17-40 passages. For Project 4, all cell lines were thawed and cultured in wells coated with human recombinant laminin 521 in NutriStem for 4-5 passages before characterization.

3.3 CELL PROLIFERATION

3.3.1 ^{3}H-Thymidine incorporation (Paper I)

To measure cell proliferation, cells were incubated for 24 hours with radioactively labeled thymidine, which is incorporated into the DNA of dividing cells. The read-out gives the amount of radioactively marked cells actively proliferating during the incubation period. The cells were stimulated with $(Bu)_2$cAMP, PDGF-A, LIF and their combinations for 72 hours prior to incubation with labeled thymidine and compared to untreated controls.

3.4 PROTEIN EXPRESSION

3.4.1 Immunohisto- and Immunocytochemistry (Paper I-IV)

Immunohistochemistry (IHC) uses specific antibodies to identify, localize and to some extent quantify protein expression in cells or tissues. The procedure was first published by Dr. Albert Coons in 1942 and improved in 1950 [135].

Depending on the fixation and staining methods used, IHC provides information about protein expression in specific cell types in tissue samples, the morphology of the tissue, as well as co-localization of different proteins.

To visualize an antigen-antibody interaction between a target and a primary antibody, two different standard methods are used. A secondary antibody conjugated with peroxidase, streptavidin or biotin can bind and oxidize 3,3'-Diaminobenzidine (DAB), producing a brown color in the vicinity of the target protein. Secondary antibodies bound to a fluorescent dye are used especially for the analysis of co-expression. By using different dyes detectable at different wave lengths the expression of several proteins can be monitored in the same sample.

To control for the specificity of the primary antibody, samples of tissues or cells with known expression patterns can be used as a positive control. Samples treated with unspecific IgGs produced from the same species as the primary antibody are used as controls for unspecific background staining.

3.4.2 Western Immunoblot (Paper I & III)

Western Immunoblot is a standard method used to identify and quantify proteins. In contrast to IHC, the result does not provide any information about the localization.

The method was developed in the laboratory of Harry Towbin in 1979 [136]. It is a semiquantitative method that can be applied for the detection of proteins in cell and tissue samples.

In a first step, the cells are lyated and the proteins denaturated. The denaturated proteins are separated according to their molecular size on a SDS-polyacrylamid gel and transferred to a membrane, which is subsequently stained for immunodetection.

Several controls are routinely included in the procedure. A mixture of standard protein with known molecular weights is added to the SDS-gel to determine the size of the immunostained protein. Staining of a reference house-keeping protein verifies equal loading of samples. A sample where the protein is known to be expressed, can be used as a positive control to confirm the specificity of the antibody.

To quantify protein expression, the density of the bands is determined and assessed relative to the house-keeping protein.

3.5 GENE EXPRESSION

3.5.1 RT-PCR (Paper I-III)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a standard method to detect and, to some degree quantify gene expression in cell- or tissue samples by *in vitro* amplification of cDNA fragments. RT-PCR was developed in 1987 by Kary Mullis, who earlier discovered Taq polymerase, the prerequisite for PCR development [137].

A PCR cycle is divided into three steps. First the sample is heated to 96˚C to denaturate the DNA strands, then the temperature drops to around 60˚C to allow specific primers to bind and initiate elongation of the complementary strand. The temperature is then raised again to 72˚C, which allows the Taq polymerase to synthesize the new DNA strand. This cycle is repeated for up to 40 times, which leads to an exponential increase in the copy number of the amplified cDNA fragment.

To verify the efficiency of the PCR and the specificity of the primer pairs, several control reactions are required. The expression of a house-keeping gene (*e.g.* β-actin or GAPDH) is determined for each sample to check for equal cDNA concentrations. A tissue or cell type sample with known gene expression level should be included as a positive control, and reaction samples prepared without reverse transcriptase can be used as negative controls to exclude amplification of chromosomal DNA. Primer specificity can be controlled by determination of the PCR product size on an agarose gel.

3.5.2 Q-PCR (Paper I-IV)

In addition to detection of gene expression, quantitative polymerase chain reaction (qPCR) measures cDNA production in real-time and can be used to compare the

gene expression between different groups, *e.g.* before and after treatment, or in different cell types or tissues, respectively.

The gene expression is normalized to the expression of a house-keeping gene and quantified with a standard curve, as done in Project 1+2, or calculated with the comparative Ct method after Livak as done in Project 3+4 [138].

Experiments described in this thesis work used Sybr Green, a dye that binds to double-stranded DNA to detect amplified cDNA strands.

Similar to RT-PCR, tissues or cells can be used as positive controls and cDNA produced without reverse transcriptase as controls to exclude chromosomal DNA amplification. To verify the specificity of the primer, the product size is checked by agarose gel electrophoresis.

3.5.3 TLDA (Paper IV)

The TaqMan low density array (TLDA) uses quantitative PCR to measure gene expression. It is based on the exonuclease assay TaqMan [139]. Briefly, the probes that consist of a fluorophore bound to the 5'-end of an oligonucleotide and a quencher on the 3'-end, anneal with a specific DNA region. After amplification of the DNA strand, the polymerase activity degradates the quencher and allows the fluorescence of the fluorophore.

In Project 4, microfluidic cards designed for stem cell research [140] that contained primers for 96 genes expressed in undifferentiated stem cells (NANOG, POU5F1, TDGFI, DNMT3B, GABRB3, GDF3), involved in maintenance of pluripotency (NANOG, POU5F1, SOX2) or early markers for differentiation, were employed.

The gene expression is normalized to the mean of six house-keeping genes to control for comparability of results.

3.6 STEROID PRODUCTION

3.6.1 RIA (Paper I-III)

The radio immune assay (RIA) is an immunological method to quantify protein/steroid production. The first RIA was developed by Rosalyn Yalow and Aaron Berson in 1960 to measure insulin in patient serum samples [141].

Radioactively labeled substrate and the samples are mixed in an antibody-coated tube. Competitive binding to the antibody between ^{125}I -radiolabeled and the present analyte occurs. A standard curve based on known concentrations of substrate is used to determine the substrate concentration in the samples.

3.6.2 Steroidogenic enzyme activity assay (Paper I)

To measure the enzyme activity, radioactively labeled steroids are added to the samples for 24 hours. After separation by thin-layer chromatography (TLC) the amount of the remaining substrate and the formed metabolites are quantified by scintillation counting.

TLC sorts steroids by polarity, *i.e*. according to their OH-groups. The samples are applied to a silica-gel coated aluminium foil and eluated with a mobile phase containing chloroform and ethylacetat in 4:1 ratio. Capillary action causes upward migration of the analytes and migration velocity is proportional to analyte polarity.

Samples can be detected with UV-light or after iodine exposure. The respective spots are cut out from the foil and their radioactivity measured by scintillation.

3.6.3 Mass spectrometry (Paper III)

Mass spectrometry (MS) measures the weight-to-charge ratio of molecules with high accuracy. A combination of liquid chromatography and MS can be used to determine the presence and identity of biological molecules such as, in this case, steroids.

Briefly, the molecules in a sample solution are separated based on polarity by reverse phase chromatography, ionized and broken down by collision with argon gas. The resulting fragments are analyzed by MS, and characteristic fragmentation patterns are used to identify the steroids present in the sample.

4 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF POSTNATAL FETAL LEYDIG CELLS (PAPER I)

To identify the stem Leydig cell, we first characterized the two steroidogenic cell types in the neonatal rat testis to elucidate the fate of fetal Leydig cells after birth and to characterize postnatal fetal Leydig cells (FLCs) according to their steroidogenic capacity *in vitro.*

FLCs produce the androgens that are responsible for the masculinization of the fetus during its development. However, their fate after birth remains to be determined. It has been suggested that they can degenerate, dedifferentiate to fibroblastic cells, persist as second steroidogenic cell type in the testis or give rise to the adult Leydig cell lineage [48, 52-54].

4.1.1 Phenotype of postnatal fetal Leydig cells

To analyze the phenotype of the postnatal fetal Leydig cells, a protocol for magnetic cell separation (MACS) of FLCs for LHR was established. With about 98% LHR positive cells, the cell population was highly enriched. Furthermore, 97-98% of the cells expressed Leydig cell specific markers such as Insl3, LHR, 3βHSD and StAR, indicating that they are fully differentiated LCs (Figure 14).

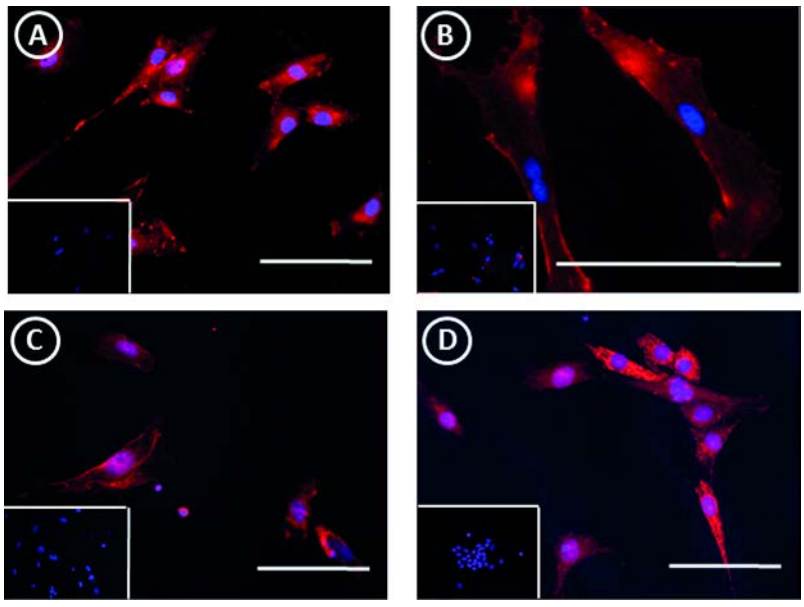

Figure 14: Expression of Leydig cell markers by FLCs; A: Insl-3, B: LHR, C: 3βHSD, D: StAR; nuclei are counterstained with DAPI. The corresponding negative controls are shown in the bottom corners. Size bar: 20µm

4.1.2 $(Bu)_2$cAMP stimulates steroid production in PFLCs

Isolated PFLCs maintained a transient capacity to produce testosterone *in vitro.* Unstimulated cells stopped to produce testosterone after 24 hours and all steroid production ceased after 72 hours. However, stimulation with hCG or $(Bu)_2$cAMP prolonged and increased steroid production.

During long-term culture FLCs stopped to express Leydig-cell related genes like 3βHSD, P450c17 and Insl-3 and showed signs of dedifferentiation that were preventable by $(Bu)_2$cAMP.

$(Bu)_2$cAMP treatment restored steroidogenic gene expression, but a Western Blot analysis revealed decreased functional protein expression of P450cyp17. Due to P450cyp17 suppression at the translational level, progesterone accumulated in the culture medium after 48 hours when testosterone production decreased.

Treatment with PDGF-A and $(Bu)_2$cAMP, individually or in combination stimulated FLC proliferation.

The fate of the fetal Leydig cell generation has been widely discussed [48, 52-54]. The results of this study showed that immediately after birth, FLCs still possessed the capacity to produce steroids, but lost this ability *in vitro* within 72 hours. The expression of Leydig cell specific markers such as Insl-3 and LHR was severely attenuated, suggesting a process of dedifferentiation. Similar observations have been made in cultured hamster Leydig cells [142] and in dedifferentiated human LCs [143].

However, stimulation with cAMP was able to rescue FLCs from dedifferentiation and induced proliferation, indicating that the cAMP-PKA pathway plays an important role in the regulation of steroidogenesis and mitotic activity in the FLCs. It had been shown previously that this pathway was involved in upregulation of genes encoding steroidogenic enzymes in mouse Leydig cells [99, 100].

PDGF-A stimulated FLC proliferation, a finding that is in accordance with previous studies, which had shown that PDGF-A signaling was active in FLCs [144], and PDGFRα-knock out studies, which showed significantly reduced FLC numbers [8].

In summary, neonatal FLCs possessed a steroidogenic capacity, which was lost under *in vitro* conditions within 72 hours due to an attenuation of functional P450c17 expression. This loss was partially rescued by cAMP stimulation. Both cAMP- and PDGF-signaling were involved in the proliferation of FLCs.

4.2 IDENTIFICATION OF PUTATIVE STEM LEYDIG CELLS IN THE NEONATAL RAT TESTIS (PAPER II)

Having identified the transient steroidogenic capacity of the postnatal fetal Leydig cells and its stimulation through cAMP and hCG, we investigated whether the

phenotype and the steroidogenic profile of the PDGFRα-positive putative stem Leydig cells exhibit similar characteristics.

Several studies have suggested that the origin of the adult Leydig cell lineage is located in the peritubular compartment of the testis. The putative precursor cells have been described as spindle-shaped steroidogenic enzyme containing cells in the peritubular region [55, 57, 60, 145]. Gnessi *et al.* reported that neonatal peritubular cells express PDGFRα and that this receptor was otherwise restricted to the Leydig cell lineage within the testis [62]. In addition, Ge *et al.* reported stem Leydig cells in 7-day old rat testes to be PDGFRα- and LIFR- positive, and LHR-negative. These cells have been described to possess a self-renewing capacity in long-term culture and began to express Leydig-cell related genes after cultivation in differentiation medium [1].

4.2.1 Phenotype of PDGFRα-positive peritubular cells

Immunohistochemical staining of 7-day old rat testis sections revealed that peritubular cells (PTCs) co-express αSMA, a known peritubular cell marker, and PDGFRα, a receptor described to be expressed by stem Leydig cells (Figure 15, A-C).

A subpopulation of the PTCs also expressed LIFR, another stem Leydig cell marker, as well as StAR and P450scc, proteins involved in steroidogenesis, suggesting a relationship with the Leydig cell lineage.

Thus, we isolated the PTC population, using the MACS technology described in Project 1, in order to further characterize the phenotype of these putative stem Leydig cells in regard to their relationship to the peritubular cell lineage and their steroidogenic capacity *in vitro*.

To achieve a higher purity and prevent a contamination with fetal Leydig cells, a second, negative purification step was included. First, the cell suspension was depleted off LHR-positive fetal Leydig cells and then PDGFRα-positive cells were isolated.

The isolated cells expressed both PDGFRα and αSMA and a subpopulation was also positive for LIFR, StAR and P450scc, a steroidogenic enzyme (Figure 15, D-F), indicating a potential steroidogenic capacity of the isolated PTCs.

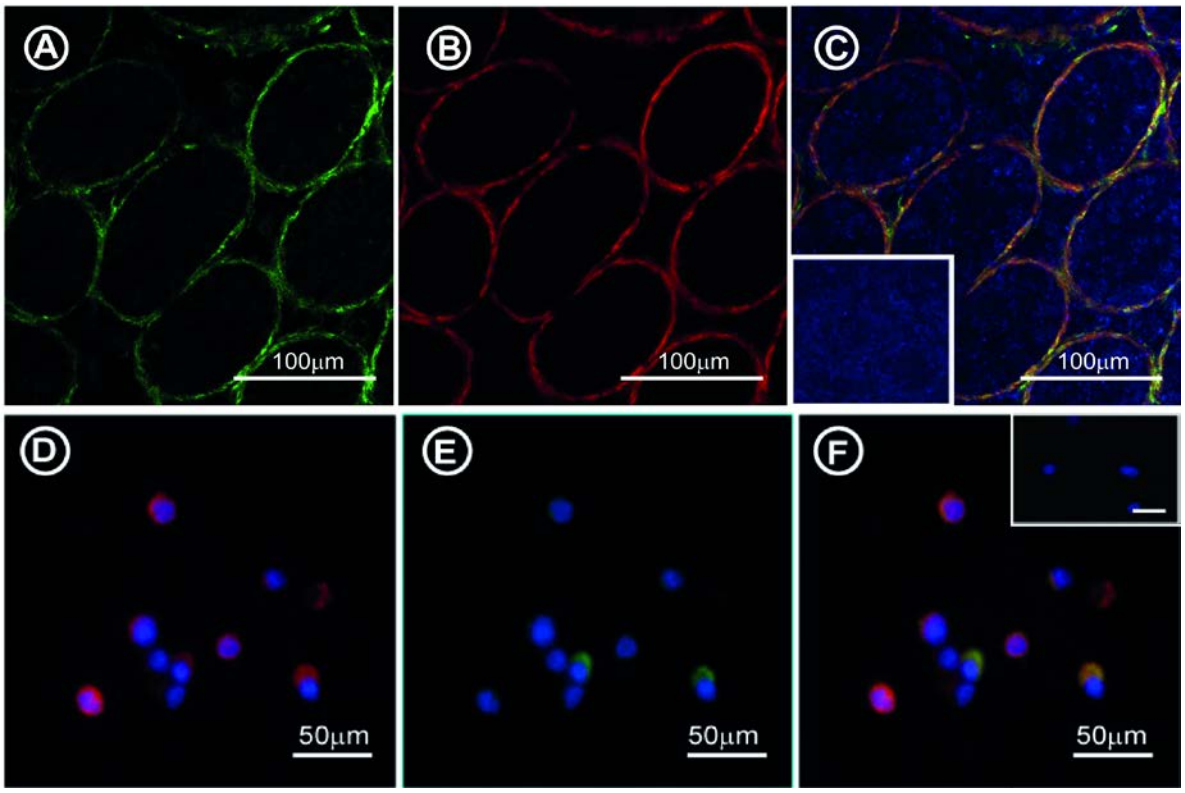

Figure 15: 7day old rat testis and isolated PTCs stained for stem Leydig cell, peritubular and steroidogenic marker. A: PDGFRα (green), B: αSMA (red), C: overlay, D: PDGFRα (red), E: P450scc (green), F: overlay. Nuclei are counter stained with DAPI. Negative controls are displayed in figure C, bottom left and figure F, top right respectively.

To further characterize the phenotype of the isolated cells, their gene expression profile was analyzed using quantitative PCR. Gene expression for Nes, Pou5f1 (markers for pluripotent cells), Lifr, Pdgfra (markers for stem Leydig cells), Myh11 (marker for peritubular cells) and Nr5a1, Tspo and Star (markers for steroidogenic cells) was detected. Furthermore, low level gene expression was identified for genes encoding steroidogenic enzymes. Expression of markers for mature Leydig cells, such as Insl3 and Lhr, was negligible. Taken together, these findings indicated that these cells were in an early stage of LC development and confirmed that the cell population was not contaminated by FLCs.

Radioimmuno assays showed that the PDGFRα-positive PTCs were able to produce very low levels of testosterone for one day and progesterone for five days. During longer culture periods, inactivation of P450c17 and 17βHSD occurred resulting in the accumulation of progesterone in the culture medium.

4.2.2 $(Bu)_2$cAMP stimulates steroid production in PTCs

cAMP has been described to be involved in the regulation of steroidogenesis in mouse Leydig cells [99, 100] and plays an important role in fetal Leydig cell steroidogenesis as shown in Project 1 [146].

To explore whether the cAMP-PKA-pathway activates steroidogenesis in putative stem Leydig cells, the cells were grown in the presence and absence of $(Bu)_2$cAMP for seven days.

Stimulation with $(Bu)_2$cAMP increased and prolonged steroid gene expression as well as steroid production in the PDGFRα-positive PTCs. It furthermore attenuated dedifferentiation towards the PTC phenotype as shown by down-regulation of the Myh11-expression.

Taken together, these findings suggest that the neonatal PDGFRα-positive PTCS are possible precursors for the adult Leydig cell lineage. In these cells, the cAMP-mediated signaling is involved in the regulation of steroidogenesis and preservation of the steroidogenic phenotype.

4.3 HUMAN PERITUBULAR CELLS CONTAIN PUTATIVE STEM LEYDIG CELLS (PAPER III)

In order to verify the transferability of the rodent data into humans, we subsequently analyzed the phenotype and the steroidogenic capacity of human peritubular cells (HTPCs).

Earlier studies have suggested that fibroblastic cells in the tubular wall are a source of differentiated Leydig cells in humans [147, 148], and that these mesenchymal cells in the testes of prepubertal boys can be stimulated to produce testosterone [147].

Electron microscopy analysis of the peritubular wall of infertile patients with cryptorchidism or Sertoli-cell syndrome has revealed the presence of mature Leydig cells [149]. In healthy tissue, differentiating Leydig cells are migrating into the interstitial space and are not found in the peritubular wall, but the authors reported the occasional presence of spindle shaped Leydig cells in between the peritubular layers [149].

4.3.1 Characterization of human testicular peritubular cells

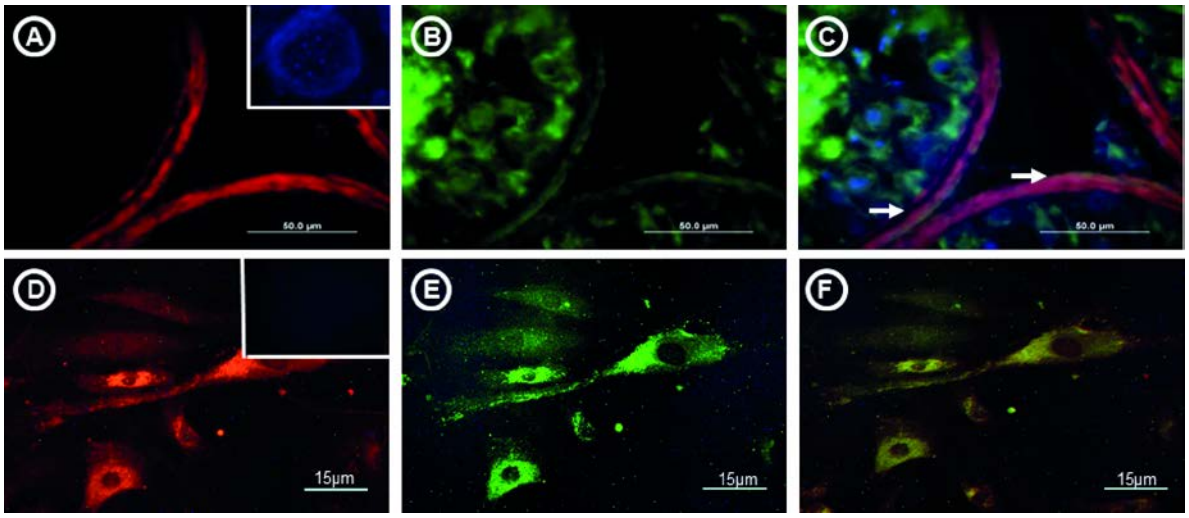

Figure 16: Adult human testis and isolated HTPC stained for peritubular, stem Leydig cell and pluripotency markers. In the testicular sections the HTPCs co-express αSMA (A) and PDGFRα (B). An overlay of αSMA and PDGFRα is shown in (C). Isolated HTPCs stained for Nanog (D) and PDGFRα (E) with an overlay (F). Negative controls are displayed in the upper right corners of A and D, respectively.

In the testis of infertile men, StAR positive cells were found in the peritubular compartment and electron microscopy showed immature Leydig-like cells containing lipid droplets and smooth endoplasmatic reticulum within the peritubular wall. These results led to the hypothesis that the HTPCs could be related to the Leydig cell lineage and possibly be stimulated to become steroidogenic.

Immunohistochemical staining revealed that there are HTPCs that co-express αSMA and PDGFRα (Figure 16, A-C), showing the same expression pattern that was found in PTCs from neonatal rat testes [150].

To further characterize this cell population, HPTCs were isolated from human testicular biopsies from adult men with obstructive and non-obstructive azoospermia. These explant cultures provided a unique *in vitro* model to examine various aspects of HTPCs. The isolated cells were found to express αSMA, a marker for peritubular cells, and PDGFRα, typical for putative stem Leydig cells. The cells stained positively for StAR, which may suggest a steroidogenic capacity. Nanog expression was found in the cytoplasm, which indicates pluripotent qualities (Figure 16, D-F).

The gene expression profile analyzed by RT-PCR confirmed the expression of markers for pluripotency, stem Leydig cells, steroidogenesis, as well as steroidogenic enzymes. No mature LC markers such as LHR and INSL3 were found.

Stimulation with PDGF-B confirmed the functionality of the PDGFRα and increased the STAR expression. Therefore, PDGF-signaling seems to play a role in the regulation of the steroidogenic phenotype.

4.3.2 Forskolin stimulates steroid production in HTPCs

Forskolin is known to activate adenylate cyclase to enhance the cAMP production and activate the cAMP-PKA signaling pathway. Forskolin stimulation of the HTPCs led to an increase in the expression of STAR and CYP11A1 as well as promotion of steroid production. This indicated that the cAMP-PKA pathway was involved in steroidogenesis in the human PTCs.

Taken together, the results of Project 3 suggest that HTPCs may be related to the adult Leydig cell lineage in humans. They possess pluripotent and steroidogenic capacities, and their steroidogenic phenotype is upregulated through cAMP-PKA signaling.

4.4 IDENTIFICATION OF HES CELL LINES WITH STEROIDOGENIC DIFFERENTIATION POTENTIAL (PAPER IV)

Human embryonic stem cell lines are a suitable tool for the study early differentiation processes. Therefore, we characterized seven different male hES cell lines (HS207, HS360, HS361, HS364, HS380, HS401 and HS420, derived at the Karolinska Institutet, Huddinge) with regard to their differentiation potential towards gonadal cell lines to identify possible candidates for steroidogenic cell differentiation.

Human embryonic stem cells (hES cells) are an important tool in the field of regenerative medicine. They have the capacity for self-renewal and can differentiate into all three germ layers. Recent studies have shown that hES cell lines differ in their expression profile and seem to have preferred differentiation pathways [151-153].

Typically, ESCs are cultured on feeder cells that help to maintain the stem cell capacity of the ESCs [112]. However, for purposes related to regenerative medicine, xeno- and feeder-free culturing conditions are needed [113, 115].

4.4.1 Characterization of seven human embryonic stem cell lines with regard to their differentiation potential towards gonadal cells

The hES cells were cultured on human recombinant laminin 521-coated wells. Laminin is a component of the natural hES cell niche and has been shown to support preservation of hESCs pluripotency and self-renew capacity [113]. Unlike feeder supported culture conditions, where ES cells grow in colonies, cells on laminin-coated surfaces spread out and form a monolayer (Figure 17).

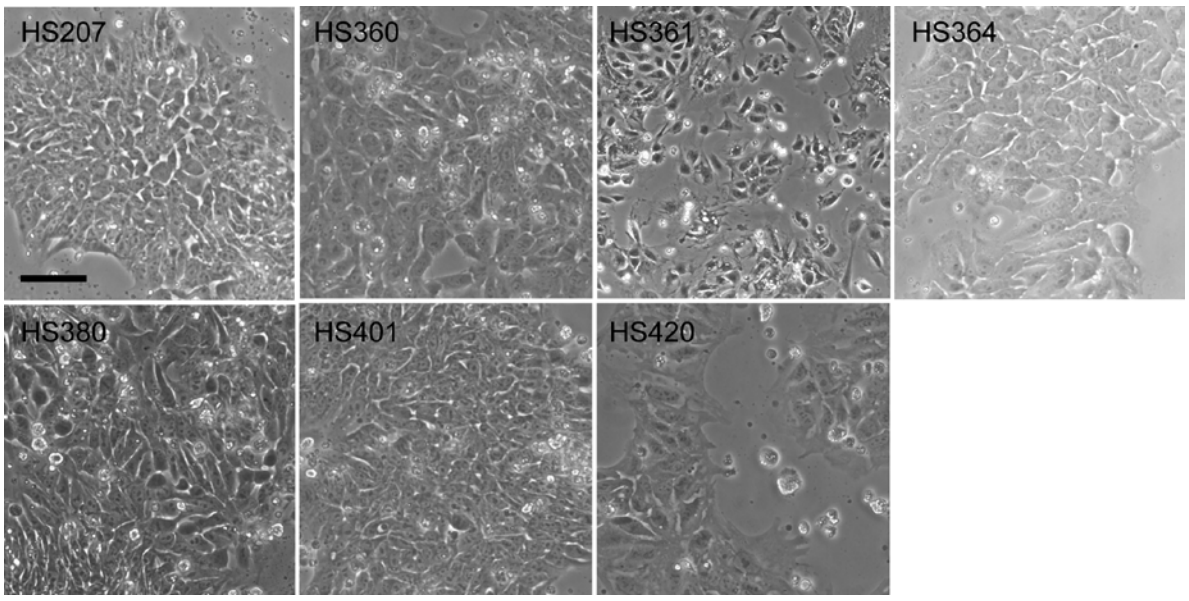

Figure 17: hES cell lines cultured on recombinant human laminin 521, size bar: 100µm

After 4-5 passages on laminin, the phenotype and the expression profile of the seven hES cell lines were analyzed. All seven cell lines had a normal male karyotype and expressed pluripotency markers on the mRNA and protein level (Figure 18).

TLDA analysis revealed differences in the expression patterns of all seven cell lines. While the expression of the house keeping genes was comparable between the samples and all seven hES cell lines still expressed the main pluripotency markers, they differed in their expression of differentiation related genes.

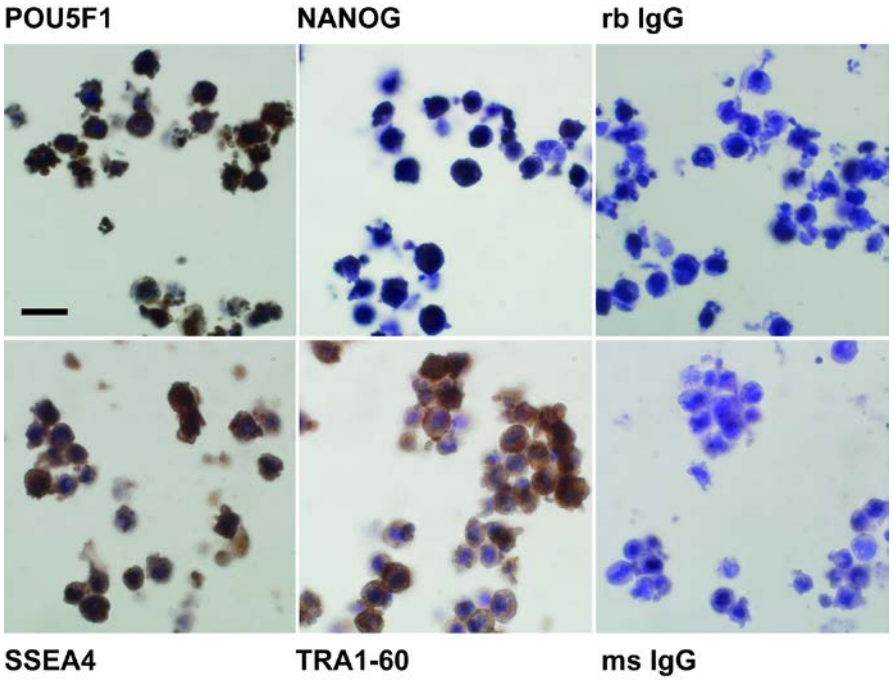

Figure 18: HS360 after five passages on LN521 expresses pluripotency marker Pou5f1, Nanog, SSEA4 and Tra1-60, size bar: 20µm

For the purpose of this study, we focused on genes that are involved in differentiation towards gonadal cell lines. As markers for germ cell development, the expression of SOX2, NODAL, SYCP3, KIT and DDX4 was taken into account. There was no cell line that expressed significantly higher levels of these genes. To identify a capacity to differentiate towards the Sertoli cell lineage, the expression of Sertoli cell related genes was analyzed, i.e. SOX17, GATA6, SOX9, FSHR, SCF, GATA4, AR and WT1. Besides AR, all other genes were upregulated in HS361, suggesting that this cell line contained more cells with Sertoli cell-like characteristics than the other cell lines.

The expression of different steroidogenic cell markers, *e.g.* NR5A1, NR5A2, STAR, CYP11A1 and HSD3B, was measured to detect cell lines with the capacity to become steroidogenic cells. HS420 contained cells that expressed higher levels of some of NR5A1 and HSD3B.

To further distinguish between the different steroidogenic cell types, the expression of HCGB was analysed. In HS420 the expression was significantly upregulated compared to all other cell lines indicating that this cell line contains cells with a differentiation potential towards trophectodermal cells. Placental cells are a steroidogenic cell type expressing βHCG and this could explain the upregulation of NR5A1 and HSD3B in this cell line. Spontaneous differentiation into trophectodermal cells in embryoid bodies has been described before [129].

We confirmed that hES cell lines differ in their expression profiles and have different dispositions towards individual differentiation pathways. Cultivation on laminin maintained the pluripotency and self-renew capacity of all seven cell lines for at least four passages. We found cell lines, *i.e.* HS361 and HS420, with a tendency to differentiate into Sertoli and steroidogenic cells.

In conclusion, we found that there are two distinguished steroidogenic cell types in the postnatal rat testis and that the peritubular cells in both, rodents and humans, contain putative stem Leydig cells. Furthermore, we showed that embryonic stem cell lines have different expression profiles and that a carefully chosen cell line could provide a starting point for further targeted differentiation studies.

5 CONCLUDING REMARKS

In this thesis, different stages of Leydig cell development and human embryonic stem cells have been analyzed to elucidate the origin of the adult Leydig cell lineage and to identify mechanisms that are involved in the differentiation towards steroidogenic cells.

The postnatal fetal Leydig cells showed to retain a capacity to produce steroids especially upon stimulation with hCG or cAMP, but lost this ability within 72 hours in culture due to an attenuation of P450c17 translation. Furthermore, signs of dedifferentiation were found under *in vitro* conditions as shown by the downregulation of steroidogenic enzyme and mature Leydig cell markers like LHR and Insl3.

Treatment with $(Bu)_2$cAMP led to an increase of steroidogenic gene expression as well as steroid production, indicating that cAMP-PKA signaling is involved in the regulation of steroidogenesis. Both, cAMP and PDGF-A stimulation, increased the proliferative activity of FLCs.

The PDGFRα-positive peritubular cells isolated from neonatal rat testis expressed markers for pluripotency, stem Leydig cells, peritubular and steroidogenic cells but no factors expressed by mature Leydig cells. This suggests that they are possible precursor cells for the adult Leydig cell lineage. Freshly isolated, the cells produce low levels of testosterone and progesterone. The production is increased upon $(Bu)_2$cAMP stimulation, which indicates that the cAMP-PKA signaling is also involved in the regulation of steroidogenesis in this cell type.

Electronmicroscopy studies support that the human testicular peritubular cells can host a Leydig cell precursor population. The HTPCs were shown to express the PDGFRα and markers for pluripotency, peritubular and steroidogenic cells, without the expression of mature Leydig cell markers. Stimulation of the cAMP-PKA signaling led to an increase in steroidogenic gene expression and steroid production after 6 and 24 hours. Furthermore, treatment with PDGF-B increased the StAR expression indicating a possible link between the PDGF-signaling and steroidogenesis.

Taken together, these findings suggest that the HTPCs are related to the adult Leydig cell lineage.

Seven human embryonic stem cell lines were analyzed for their differentiation potential towards gonadal cells. HS361 expressed higher levels of Sertoli cell markers than the other six cell lines, and HS420 expression patterns resembled more steroidogenic cell expression patterns, but also included the placental cell marker HCGB. To draw conclusions about mechanisms underlying steroidogenic cell differentiation, a continuation of the project with successful differentiation towards the Leydig cell lineage would be needed.

In Project 1 and 2, rat testicular cells were studied. Rodents and rats especially are commonly used for studies of testicular structure and function. Their morphological features are comparable to most other mammalian species, and rats are the most common animal model in reproduction and toxicology studies. However, conclusions from animal experiments need to be validated in humans.

Due to obvious ethical reasons, the possibilities to study human material are limited. The outgrow cultures of peritubular cells from human testicular biopsies as described in Project 3 are a useful model to study various aspects of human testicular peritubular cells and offer a unique possibility to validate the findings of Project 2 in human cells.

The results of this work provide new insights into the origin of the adult Leydig cell lineage. PDGFRα-positive peritubular cells are possible precursors for ALCs and the cAMP-PKA pathway is involved in the regulation of steroidogenesis and differentiation towards steroidogenic cells.

All findings of this work originate from *in vitro* studies and need to be validated in *in vivo* studies before clinical implications can be considered.

The identification of differentiation mechanisms for gonadal cells and the exploitation of pluripotent stem cells in the recreation of testicular tissue may become an important approach to (re)-establish fertility in sub- or infertile men.

6 FUTURE PERSPECTIVES

Overall, there seems to be a relationship between the two generations of Leydig cells and the peritubular cell lineage. The cAMP-PKA pathway plays an important role in the regulation of steroidogenesis in all three cell types and PDGF signaling is involved in the regulation of their proliferation and steroidogenesis.

Human ES cell lines have the potential to differentiate into all cell types of the human body, mechanisms underlying the differentiation into gonadal cell types have to be further explored to understand the development of the testis.

To further analyze the fate of fetal Leydig cells and to elucidate their possible role in the adult testis, it would be interesting to examine the mechanisms of their dedifferentiation and to find signaling pathways to prevent it. To study FLC behavior *in vivo*, isolated fetal Leydig cells could be transplanted into Leydig cell depleted rat testes after EDS treatment and their capacity to repopulate the interstitium and to produce steroids could be studied.

Our work provided evidence that the peritubular cells are a possible source of the adult Leydig cell lineage. We found characteristics of pluripotent and steroidogenic cells in our isolated PDGFRα-positive cell population. To confirm these results *in vivo*, experiments similar to those described for the FLCs should be performed, including transplantation of GFP positive PTCs after EDS treatment.

Another aim for a future study of peritubular cells and their relation to the Leydig cell lineage is successful differentiation of peritubular cells into testosterone producing mature Leydig cells. Short-time stimulation of the cAMP-PKA pathway led to an increase in pregnenolone and progesterone production as well as to steroidogenic gene expression, but no testosterone was produced. PDGF-B stimulated StAR expression in the HTPCs after 24 hours, but did not further increase steroidogenesis.

A promising study by Ge *et al.* described differentiation of rat stem Leydig cells into 3βHSD-positive cells grown in differentiation medium containing a combination of factors including LH, IGF-1, T3 and PDGF [1]. However, testosterone production of these cells was very low [1] and these results need to be confirmed for human peritubular cells.

Our understanding of differentiation pathways underlying development of gonadal cells is still limited. After identification of the possible hES cell lines as candidates for different gonadal cell types, their differentiation capacity into the different cell lines needs to be further defined.

Different experimental approaches, such as spontaneous differentiation in embryoid body formation, directed differentiation following published differentiation protocols, and the targeted induction of differentiation pathways, such as the cAMP-PKA pathway in the differentiation towards steroidogenic cells, could be compared.

Differentiation success has to be confirmed by functional studies and transplantation of differentiated cells.

To be able to help patients suffering from infertility, a lot of information about the development of the gonadal cells has yet to be collected. Being able to use stem cell technology to create all testicular cell types *in vitro* could help to identify differentiation mechanisms and to restore spermatogenesis and steroidogenesis. This knowledge could be used to re-establish fertility-potential in a subpopulation of infertile men.

Male survivors of childhood cancer having undergone gonadotoxic treatment or of other severe systemic diseases are an increasing group of patients that will profit from this research in the future.

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